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TITLE: STRUCTURAL CHARACTERIZATION OF CROSS-LINKED HEMOGLOBINS DEVELOPED AS POTENTIAL TRANSFUSION SUBSTITUTES

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Structural and functional studies of chemically modified hemoglobins have been done in an effort to find reagents and conditions that are suitable to produce cross-linked hemoglobin derivatives that would be suitable for use as acellular substitutes for the transfusions of erythrocytes. The effects of modifying hemoglobin with five different dicarboxylic bis(methyl phosphate) reagents provided by Professor Ronald Kluger have been examined by ion exchange separation of hemoglobin products, globin chain separation, peptide pattern analysis, and oxygen equilibrium measurements. These reagents were found to react only with β1val, β82lys, α1val, α99lys, and α139lys but to varying extent depending on the conformational state of the hemoglobin. Both cross-linked and uncross-linked products have been found. Several have reduced oxygen affinities. The hemoglobins present in Batch 11 of DBBF-Hb from Baxter was examined by the same chromatographic and structural procedures in order to determine the extent and structural basis of the heterogeneity of this product.

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Richard T. Jones   May 15, 1992

PI Signature   Date

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REPORT

1. INTRODUCTION

A. The Goal and Objectives of Study:

The goal of this study is to test the hypothesis that it is possible to produce in high yield, chemically cross-linked hemoglobin derivatives that will not dissociate into subunits and that will have oxygen binding, transport, and delivery properties which make them suitable and practical for use as acellular substitutes for whole blood in transfusions and perfusion of isolated organs. This goal is being perused by means of the following objectives: 1) carry out structural characterization of the specific modifications of chemically cross-linked hemoglobins; 2) correlate changes in oxygen binding properties with specific chemical modifications; 3) design and have synthesized bifunctional reagents that will react more specifically with hemoglobin; and 4) test these and other cross-linking reagents to optimize the reaction conditions in order to obtain maximum results.

B. Background:

The desirability of a transfusion substitute for whole blood that will transport and deliver adequate amounts of oxygen at physiologically appropriate pressures of oxygen has been recognized and reviewed by others (1,2,3,4). A hemoglobin based oxygen transport product could have the following advantages over whole blood or packed red cells: longer shelf-life, simpler storage and transport requirements, no need for blood typing and cross-matching, and elimination of problems of transmission of viruses such as those for hepatitis and AIDS. Additionally, hemoglobin solutions would have oncotic activity that could be used to maintain or expand blood volume and should perfuse capillary beds more readily than red cells. Such features as these make solutions of hemoglobin attractive candidates for rapid, initial treatment of hypovolemia and tissue hypoxia and could have applications for perfusion of specific organs *in vivo* and *in vitro*.

Several significant problems exist that make the transfusion of acellular, unmodified hemoglobin solutions impractical. First, native hemoglobin is cleared rapidly from the vascular system (5). This is due to the dissociation equilibrium between tetramers and dimers of hemoglobin subunits and the fact that the dimers are readily filtered by the glomerulus of the kidney if the subunits are not associated with haptoglobin. Second, native hemoglobin in plasma, outside the erythrocyte, has a high intrinsic oxygen affinity due mainly to the virtual absence of 2,3-diphosphoglycerate (DPG). This high affinity for oxygen diminishes its unloading in the tissues at a physiologically acceptable partial pressure (6). Third, when iron of the heme groups is oxidized to the ferric form, the resulting methemoglobin subunits will not bind and transport oxygen. Methemoglobin once formed in plasma outside the erythrocyte will not be reduced to functional hemoglobin because of the lack of appropriate reducing potential and enzymes that are normally present only in the erythrocyte (6). Finally, early attempts to use hemoglobin solutions were associated with adverse effects on renal function and the coagulation system. These effects, found to be due to contaminants from the red cell stroma (7,8,9), appear to have been overcome by preparation of stroma free hemoglobin solutions (10).

One approach to the problem of the high intrinsic oxygen binding property of extracellular hemoglobin has been to chemically modify hemoglobin in ways that favor the low oxygen affinity state or "tense" (T) conformation of the molecule (6,11). This was first accomplished by Benesch et al (12) who demonstrated that pyridoxal phosphate (PLP) has an analogous effect on oxygen affinity as does DPG and can be attached covalently to the N-terminal group of the beta chain of hemoglobin. However, the intravascular retention of this modified hemoglobin was found to be similar to unmodified hemoglobin (13). The problem of dissociation into dimer subunits has been approached by attempting to cross-link the tetramer with several different bifunctional reagents (reviewed in 14). Two such cross-linking reagents that have been used are glutaraldehyde (14,15) and 3,5-dibromosalicyl-bis fumarate (DBBF) (16,17). When reacted with oxyhemoglobin, most of these cross-linking reagents result in modified hemoglobins with increased affinities for oxygen compared to unmodified hemoglobin (18). Several investigators have approached the subunit dissociation and high oxygen affinity problems by combining cross-linking reactions with pyridoxylation to obtain stable tetramers or aggregated hemoglobins with low oxygen affinities (1,3,4,14,17,19,20). These combined modifications have the disadvantage of requiring two or more chemical reaction steps, including a reduction with sodium borohydride or similar reagents, and result in the formation of heterogeneous hemoglobin products (21). Other investigators have tried to obtain cross-linked hemoglobins with lower than normal oxygen affinities using single, bifunctional reagents. Benesch et al (22) were the first to show that hemoglobin could be cross-linked with 2-nor-2-formylpyridoxal 5'-phosphate (nfPLP) to form a modified hemoglobin that binds oxygen cooperatively with a greatly decreased affinity. This nfPLP-Hb derivative has properties satisfactory for transfusion purposes; however, it is considered by some to be an impractical approach because nfPLP cannot be synthesized easily (17). Nevertheless, Benesch et al (23) have continued to pursue this approach to the chemical modification of hemoglobin. Attempts to use other dialdehyde cross-linking compounds like periodate oxidized ATP (o-ATP) by Scannon (24) and Greenberg and Maffuid (25) have been found by Kavanaugh et al (26) to result in heterogeneous hemoglobin products with low yields of a cross-linked hemoglobin which has an increased rather than a decreased affinity for oxygen compared to unmodified hemoglobin. Chatterjee et al (27) have reported a new hemoglobin derivative cross-linked between the alpha chain subunits at the two $\alpha 99$ lysyl residues when deoxygenated hemoglobin is reacted with 3,5-dibromosalicyl-bis fumarate (DBBF). Preliminary studies of other cross-linking reactions by Kavanaugh (28) using trans-4,4'diisothiocyanostilbene-2,2'-disulfonate (DIDS) indicate that it may be possible to select or design specific reagents that will react directly with hemoglobin and not require additional reaction steps to form stable tetramer hemoglobins with oxygen affinities that are in a physiologically useful range.

C. Rationale Of Present Study:

Before 1989 very little exact structural data of presumably cross-linked human hemoglobins had been reported except for structural studies by Arnone et al (29) on nfPLP-Hb, Chatterjee et al (17) on the alpha chain cross-linked hemoglobin ($\alpha 99$-fumarate-$\alpha 99$), and Kavanaugh et al (21,26,28). It is essential that the exact structural modifications of chemically cross-linked hemoglobins be determined in order that the information can be used for designing and developing better modifying reagents and to improve the reaction conditions for chemically modifying hemoglobin for transfusion purposes. Equally important to the design of better cross-linked hemoglobins is information about the oxygen binding properties of each chemically modified hemoglobin. Taken together the structural and functional data can be used to predict more accurately the probable results of changing the dimensions, geometry, and functional groups of new potential cross-linkers.

Results obtained by Kavanaugh et al (21,26,28) from studies of chemical alterations of hemoglobin modified with cross-linking agents together with work published by others have demonstrated that it is feasible to rationally design a molecular engineering scheme for specific alterations of hemoglobin structure and function. With a knowledge of the atomic coordinates of hemoglobin obtained from X-ray diffraction studies, the chemistry and geometry of the functional groups in the heterotropic ligand, 2,3-DPG, and the structural changes known to occur in the $\beta 1 \beta 2$ cleft during the R to T state transition, it has been possible to design and synthesize novel cross-linked, low affinity hemoglobin, namely Hb(DIDS)2T (28). An important result obtained from studying the chemical modification of hemoglobin with DIDS is the recognition that linking the two β-N-terminal residues in the T or deoxy conformation will limit the extent of transition of the modified hemoglobin to the R conformation upon oxygenation.

A collaboration with Professor Ronald Kluger of the Department of Chemistry of University of Toronto initiated in 1988 has resulted in him and his associates designing and synthesizing several cross-linking reagents that are analogues of

3,5-dibromosalicyl-bis fumarate (DBBF), 2-nor-2-formylpyridoxal 5'-phosphate (nfPLP), and trans-4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) (28). Structural and functional studies of modified hemoglobins obtained using these bifunctional reagents of Kluger and structural characterization of a DBBF-Hb preparation are presented in this midterm report.

2. MATERIALS AND METHODS:

A. Reagents: A sample of DBBF-Hb was obtained from Dr. Mario Marini of the Letterman Army Institute of Research of the Presidio of San Francisco. This was from Batch 11 prepared and supplied by Baxter Healthcare Corp. (Round Lake IL) under contract from the United States Army Medical Research and Development Command. Stroma-free human hemoglobin was modified using 3,5-dibromosalicyl-bis fumarate according to the method of Chatterjee et al (27) as described further by Snyder et al (30). HPLC grade acetonitrile and water were obtained from Mallinckrodt and trifluoroacetic acid (TFA) was from Pierce Chemical Co. (Rockford, IL). Trypsin from Worthington Biochemical Co. (Freehold, NJ) and the staphylococcus aeries V8 endopeptidase Glu-C from Boehringer Mannheim Biochemical (Indianapolis, IN) were used for enzyme hydrolysis. Reagents for oxidation and aminoethylation of globin chains were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO). Reagents for amino acid analysis were from Pierce Chemical Co. and from Aldrich Chemical Co. Inc. DEAE-Sephacel and CM-Sephadex were from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Other reagents for preparation of buffers and developers for chromatography were all of analytical grade or better.

Professor Kluger has supplied the bis(methyl phosphates) of the following dicarboxylic acids: fumaric, isophthalic, terephthalic, trans 3,3'-stilbene dicarboxylate, and trans 4,4'-stilbene dicarboxylate. They were prepared as described by Kluger et al (31).

B. Equipment: Four separate high performance liquid chromatography (HPLC) systems are being used for these studies. Analytical and preparative separations of hemoglobins are done by ion exchange using a Beckman/Altex model 332 gradient liquid chromatograph with a model 421 microprocessor system controller, two model 110A single-piston reciprocation pumps with a dynamically stirred gradient mixing chamber, and a model 210 syringe-loading sample injection valve. The detector is a Schoeffel Instrument Corp. Spectroflow monitor model SF770 with a model GM770 monochromator. This is connected to a Brinkmann Servogor S model 2541 linear potentiometric recorder. The signal from the detector can also be recorded and processed by an IBM System 9000 computer described below. Effluent is collected with a Gilson FC-80K micro fractionator.

Analytical and preparative separations of globin chains are done by reversed phase HPLC using another Altex model 332 gradient liquid chromatograph like the one described above but with a model 420 microprocessor system controller. The detector for this system is a Hitachi model 100-30 variable wavelength (visible/UV) unit equipped with an 8μl analytical flow cell connected to a Hewlett Packard model 3392A integrating recorder. The detector signal can also be recorded and processed by the IBM System 9000 computer. The effluent is collected with a Gilson FC-80K micro fractionator in a chemical hood.

Separation and isolation of peptides are done by reversed phase HPLC using an IBM Instruments Inc. model LC 9533 ternary liquid chromatograph attached to a model F9522 fixed UV module and a model F9523 variable UV module. The detector signals are recorded by and the HPLC system is controlled by an IBM Instruments Inc. System 9000 computer which can record and process four signals simultaneously. The column effluent is collected with a Gilson FC-80K microfractionator in an externally vented box.

Amino acid analyses are done by separation and quantitation of phenylthiocarbamyl amino acid derivatives using a second IBM model LC 9533 ternary liquid chromatograph also equipped with one fixed UV detector and one variable UV detector. The signals are also recorded and integrated by the IBM system 9000 computer which controls the HPLC and an IBM Instruments Inc. model LC 9505 automatic sampler.

The hemoglobin present in the effluent from large preparative liquid chromatograms run in the cold room is detected with a Beckman Model 153 analytical UV detector connected to a Kipp and Zonen model BD 40 potentiometric strip chart recorder.

C. Procedures:

i. Hemoglobin Preparation: Fresh blood is obtained by venipuncture from normal volunteers after obtaining informed consent. Hemoglobin solutions are prepared by the method of Drabkin (32), treated with carbon monoxide (CO), equilibrated with deionized water by either dialysis or passing though a Sephadex G50 column, stripped of all the remaining ions by passing through a deionization column (33), and stored at 0°C on ice. The concentrations of hemoglobin solutions are determined spectrophotometrically, generally as the cyanmethemoglobin derivative (34).

ii. Chemical Modification of Hemoglobin: Chemical modification of hemoglobin is done using hemolysate diluted with 0.1 M bis-Tris HCl buffer at pH 7.2 to 1 mM Hb (tetramer) and cross-linking reagent generally at 2 mM unless otherwise specified. The temperature of the reaction is 35°C unless stated otherwise and the duration of reaction is 2-3 hours. Reactions are run with hemoglobin in the carbon monoxide form (COHb) or deoxygenated form (deoxyHb) without 2,3-diphosphoglycerate (DPG) or with it at 5 mM concentration. Carbon monoxide is removed by photoirradiation under a stream of humidified O_2 for sixty minutes at 0°C (35). The hemoglobin is then removed by passing a stream of humidified N_2 gas over the hemoglobin solution for 3 hours at 35°C in a closed rotary apparatus. The cross-linking reagents are dissolved in deionized water and deoxygenated under vacuum followed by bubbling with N_2 for ten minutes before using. In the case of reactions with deoxyHb, the reagents are introduced into the reaction vessel anaerobically to the desired concentration and N_2 is flushed continuously through the rotary apparatus to maintain the hemoglobin in the deoxy state. For study of reaction kinetics, aliquots are removed anaerobically at various times. The reaction is stopped by either immediately passing the reaction mixture through a Sephadex G 50 column equilibrated with the appropriate buffer or adding glycine to a concentration of 1 M and allowing the mixture to stand at 0° C for 12-18 hours before gel filtration.

iii. <u>Preparative Isolation of Hemoglobin by Liquid Chromatography:</u> The isolation and purification of single hemoglobin components in amounts of more than a few mgs is done by standard ion-exchange chromatography using DEAE-Sephacel (36) and CM-Sephadex (37). Dilute hemoglobin solutions are concentrated by ultrafiltration, stored in the CO form on ice to minimize methemoglobin formation and denaturation.

iv. <u>Analytical and Preparative Separations of Hemoglobins by Ion Exchange HPLC Procedures:</u> Hemoglobins are separated by three different ion exchange HPLC procedures. For analytical purposes a 20 x 0.46 cm column of 5 μm microparticulate poly(aspartic acid) silica packing (PolyCAT A, Custom LC of Houston, TX,) is used according to the procedure of Ou et al (38). Another cation system in use for both analytical and preparative separations hemoglobins is with a SynChropak CM300 column (250 x 4.1 mm for analytical and x 10 mm for preparative from SynChrom, Inc., Linden, IN) using developers containing 15 mM Tris-acetate at pH 8.0 and various gradients of sodium acetate starting at 10 mM and ending at 150 mM after procedures described by Huisman (39). Separations of hemoglobins by anion exchange HPLC is done with a SynChropak AX300 column (250 x 4.1 mm for analytical and x 10 mm for preparative) using developers 30 mM bis-Tris pH 6.4 and various gradients of sodium acetate starting at 30 mM and ending at 300 mM (39). The effluent is monitored at 420 nm. Dilute hemoglobin solutions are concentrated by ultrafiltration using either a magnetically stirred pressure ultrafiltration chamber or Amicon centriflo membrane cones CF25 25,000 MW (Amicon Corp., Danvers, MA).

v. <u>Analytical and Preparative Separation of Globin Chains by Reversed Phase HPCL:</u> Heme and the globin chains are separated by reversed phase HPLC using 330 Å pore size C_4 Vydac columns (250 x 4.6 mm for analytical and 250 x 12 mm for preparative, The Separations Group, Hesperia, CA) and developers containing 0.1% TFA and various gradients of acetonitrile starting at 20% and ending at 60% modified after the procedure of Shelton et al (40). The effluent is monitored at 220 nm and the globin chains are recovered from the effluent by lyophilization.

vi. Chemical Modifications and Enzyme Hydrolysis of Globin Chains: For some studies cysteinyl residues are either oxidized to cysteic acid with performic acid (41) or aminoethylated according to the procedure of Morrison et al (42). Globin chains either with or without cysteinyl residues modified are hydrolyzed with trypsin (Worthington) carried out at room temperature (25° C) in 80 mM ammonium carbonate buffer at pH 8.5 for 18-24 hours with a ratio of trypsin to globin of 1:50 by weight. In some cases, the tryptic hydrolysis is followed by heating in boiling water for 2 minutes followed by hydrolysis with endoproteinase Glu-C from staphylococcus aeries V8 (Boehringer Mannheim Biochemical) at room temperature at pH 8.5 for 12-24 hours. During the course of these studies it has been found that more complete hydrolysis of some of the more resistant globin chains preparations can be obtained if the hydrolysis was carried out in the presence of either urea or sodium dodecyl sulfate (SDS). Our current procedure is to dissolve the globin in 8 M urea and allow this to stand at room temperature for 2-4 hours. This is then diluted to 2 μ urea with 80 mM ammonium carbonate buffer and hydrolyzed with trypsin (2% by weight) for 18-20 hours at room temperature. The tryptic hydrolysate is then heated in boiling water for 2 minutes, diluted to 1 M urea with 80 mM ammonium carbonate buffer and hydrolyzed with endoproteinase Glu-C (1% by weight) for another 18-24 hours at room temperature. The hydrolysates are centrifuged or filtered through a 0.45 μm filter before injection onto the HPLC column.

vii. Separation of Peptides by Reversed Phase HPLC: Peptide fragments are separated for both analytical and preparative purposes by HPLC procedures using reversed phase C_{18} columns (25 x 0.46 cm Vydac, The Separations Group, Hesperia, CA. Cat. # 218TP54.6). In some cases rechromatography is done with an ODS Ultrasphere C_{18} column (25 x 0.45 cm Altex/Beckman Instruments Berkley, CA). Most separations are now made using developers of 0.1% TFA and gradients of acetonitrile starting at 0% and ending at 100% generated over a period of up to 100 minutes. This has been modified after the procedure of Shelton et al (43). The typical gradient for separation of α-chain peptides starts at 0% acetonitrile and changing to 13.6% by 20 minutes, then to 34% by 70 minutes, and 100% by 75 minutes. The typical gradient for β-chain peptides starts at 0% acetonitrile and changes to 12.5% by 10 minutes, 25% by 60 minutes, 50% by 100 minutes, and 100% by 105 minutes. A second developer system with 10 mM ammonium acetate buffer at pH 6.0 and acetonitrile concentration gradients is used in some cases for initial separation but more often now for rechromatography. It is adapted from the procedures of Shroeder et al (44) and Wilson et al (45). In all cases, the effluent is monitored at 214 nm which detects most peptides and also at either 280 nm to detect tyrosyl and tryptophanyl containing peptides or at 306 nm to detect peptides containing stilbene groups or 257.7 nm for the phthalyl group. Solvents and volatile solutes are removed from peptides in the effluent by lyophilization or vacuum evaporation.

viii. Amino Acid Analysis: Peptides are hydrolyzed in evacuated tubes using 6 M HCl vapor at 110°C for 22 hours. In some cases a hydrolysis time of 48 or 72 hours is used if a Val-Val or other resistant bonds are present. The amino acids are derivatized with phenyl isothiocyanate and the resultant phenylthiocarbamyl amino acid derivatives are separated by reversed phase HPLC using an IBM oxtadecyl silane column (IBM Instruments Inc. Cat. # 8635308) (46). Effluent is monitored at 254 nm and the signal is recorded and integrated with the IBM Systems 9000 computer.

ix. Polyacrylamide Gel Electrophoresis: The extent of cross-linking of globin chains is determined by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) according to the procedure of Laemmli (47). A 15% polyacrylamide gel that is 2.7% cross-linked is used. The hemoglobins and globins from HPLC separations are prepared by heat denaturation in a buffer containing 65 mM Tris-HCl pH 6.8, 2% SDS, 10% v/v glycerol, and 5% v/v 2-mercaptoethanol. Approximately 5-20 μg of protein is applied to the gel and electrophoresed at 20 mA for about 8 hours (21).

x. Measurement of Functional Properties of Isolated Hemoglobins: The hemoglobin-oxygen equilibrium properties of modified hemoglobins are measured with an automatic recording method of Imai et al (48). The data are analyzed according to the Adair stepwise oxygenation scheme described by Imai (49) using apparatus and an on-line laboratory computer described by Shih and Jones (50). The conditions for the present studies are 50 mM bis-Tris, pH 7.4, 0.1 M Cl^-, 25° C, and 55μM heme. Parameters measured are the O_2 pressure for half saturation (P_{50}) and Hill's coefficient of cooperativity at half saturation (n).

3. RESULTS AND DISCUSSION:

PART I: STUDIES OF HEMOGLOBINS MODIFIED WITH DICARBOXYLIC BIS(METHYL PHOSPHATE) REAGENTS FROM PROFESSOR KLUGER:

A. Reaction Conditions and Separation of Hemoglobin Reaction Products by Ion Exchange HPLC:

The influence of the ligand state of the heme groups, the presence and absence of 2,3-DPG, time of reaction, concentration ratio of reagent to hemoglobin, and the type or cross-linking reagent have been examined at least at the level of ion exchange HPLC profile and globin chain separation by HPLC.

Three different ion exchange HPLC methods have been tested for the assessment of the hemoglobin products resulting from the reaction of human hemoglobin with the various dicarboxylic bis(methyl phosphate) reagents of Kluger. Although excellent separations were obtained with the cation HPLC Polycat-A procedure (38), there were some problems with reproducibility and it was not practical to use it for preparative purposes. The cation HPLC SynChropak CM 300 procedure was found to be useful for both analytical and semipreparative separation of modified hemoglobins but did not resolve as many hemoglobins as the anion HPLC SynChropak AX 300 procedure did. The latter has been used for routine analytical assessments of the hemoglobin reaction products and for semipreparative isolation of some modified hemoglobins.

i. Influence of Reaction Conditions and Ligand State of Hemoglobin on Reaction Products Formed: The effect of pH, type of buffer, and ligand state of the hemoglobin has been examined in detail for the reaction of the fumaryl bis(methyl phosphate) (FMP) reagent with hemoglobin. No significant differences were observed in the amounts or varieties of reaction products when reacting COHb with FMP in bis-Tris compared to HEPES [4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid] at pH 7.5, 35° C, for 2 hours. Over the range of pH from 6.8 to 7.5, the greatest extent of reaction between COHb and FMP was obtained at pH 7.2 in bis-Tris. From these and similar studies of other cross-linking reagents and ligand states, reaction conditions have been standardized for most of the studies to: 1 mM Hb, 2 mM cross-linking reagent, pH 7.2 in 1 M bis-Tris, 35° C for 2 to 3 hours.

The number, chromatographic elution positions, and relative amounts of hemoglobin products obtained for hemoglobin reacted with Kluger's reagents vary with the ligand state of the hemoglobin and with the presence or absence of 2,3-DPG. An example of this is shown in **Figure 1** for the reaction of FMP with deoxyHb in the absence of 2,3-DPG (Figure 1a), deoxyHb with 5 mM 2,3-DPG (Figure 1b), and COHb (Figure 1c). The elution times for the unmodified Hb A vary for these three chromatograms because they were run at different times and with different lots of developers; however, the relative positions of the hemoglobins eluted are comparable. From studies described below, we have shown that the first peak in each case is unmodified Hb A. The second major peaks for each are different from one another. The third major peak for the deoxyHb-DPG preparation contains a hemoglobin that is one of several modified hemoglobins found in the third major peak of the deoxyHb preparation with out DPG. The main hemoglobin in the second major peak of the COHb preparation is found in only small amounts in the third peak of the deoxyHb preparation. The major hemoglobin in the third peak of the COHb reaction mixture is the same as that in the fifth peak of the deoxyHb mixture. The structural identities of all of these are described later. Comparable differences between the reaction products for CO and deoxyHb conditions have been found for the other dicarboxylic bis(methyl phosphate) reagents of Kluger. The effect of the presence of DPG on the reaction of deoxyHb with the isophthalyl bis(methyl phosphate) (IPMP) reagent has been studied and shown to be similar to that for FMP.

ii. <u>The Effect Time of Reaction on Products Formed:</u> Preliminary studies of the effect of time on the extent of reaction has been done for a preparation of COHb treated with FMP. The reaction at pH 7.2, 35° C, with 1 mM COHb and 2 mM FMP resulted in rapid conversion of the Hb A to at least two main products as shown in Figure 1c. By 10 minutes of reaction only 40% of the hemoglobin remained as an unreacted component and this diminished gradually to about 20% by 3 hours as seen in **Figure 2.** Although further studies of the kinetics of the reaction of these cross-linking reagents must be made with deoxyHb, these preliminary studies indicate that some modified hemoglobins are formed rapidly, with in the first 10 minutes, where as others are formed more slowly. For purposes of the present studies reaction times of 2 to 3 hours appear to be adequate to obtain sufficient modification of hemoglobin for structural studies of the major products produced.

iii. <u>The Effect of the Concentration of Cross-linking Reagent:</u> The effect of varying the ratio of concentration of terephthalyl bis(methyl phosphate) (TPMP) to a fixed concentration of deoxyHb has been studied by running the reaction at 35° C, 0.1 M bis-Tris at pH 7.2 for 3 hrs with 1 mM deoxyHb and 2, 6.7, and 20 mM TPMP reagent. **Figure 3** shows the SynChropak AX 300 anion exchange HPCL separations for the three different reagent concentrations. The percentages of unmodified hemoglobins were 58%, 19%, and 3% in order of increasing TPMP concentration. The profile of the modified hemoglobins also changed over this range of reagent concentration. From an examination of the modified globin chains which is described below, it is evident that relative amounts of different modified hemoglobins change with the concentration of TPMP used in the reaction. Although further studies of the affect of reagent concentration on the relative yields of different modified hemoglobins are necessary, the concentration of 1 mM Hb to 2 mM cross-linking reagent has been used for most of the studies directed at identifying the structures of the major modified hemoglobins produced with each of Kluger's reagents.

iv. <u>The Effect of Structure of the Cross-linking Reagent:</u> Comparison to the chromatographic behavior of the modified hemoglobins produced with different dicarboxylic bis(methyl phosphate) reagents of Kluger are shown in **Figure 4**. The bis(methyl phosphates) of fumaryl (FMP), isophthalyl (IPMP), and meta or 3,3'-stilbene dicarboxylate (MSMP) reagents were reacted at a concentration of 2 mM to 1 mM deoxyHb. The chromatogram shown for the terephthalyl (TPNP) reagent was reacted at 6.7 mM TPMP because the 2.0 mM concentration resulted in relative low product formation (see Figure 3 and discussion above). The separations were made using an analytical SynChropak AX300 anion exchange HPLC column. Although the same column, developer formulation, and gradient were used, variations in the elution times of the unmodified Hb A is probably due to small differences in conditions. The elution positions of the modified components relative to the unmodified Hb A are comparable. On the basis of structural characterization described later, the structures of some of the modified hemoglobins are shown for each chromatogram. Zone 1 in all cases is unmodified Hb A. The second zone in all cases contains a modified hemoglobin with the structural formula of $\alpha_2\beta$1-X-82β where β1 represents the Valyl residue 1 of one beta chain, 82β represents the Lysyl residue 82 of the other beta chain, and X represents any one of the four kinds of cross-linking dicarboxylic acids in diamide bonds. Zones 3 for FMP; 3 and 4 for TPMP and MSMP; and 2, 3, 4, and 5 for TPMP contain mixtures of modified hemoglobin components which are not completely separated. One prominent component in this mixture has the formula $\alpha_2\beta$82-X-82β and is the main cross-linked hemoglobin found when each of these reagents is reacted with COHb. A number of other modified hemoglobins have also been isolated and characterized from these zones. Some may be hybrid molecules with two different types of beta chains per hemoglobin tetramer and others have modified alpha chains. Some of the major modified hemoglobins that result from the reactions with each different reagent are not cross-linked. Two important examples are $\alpha_2(\beta1\text{-X})_2$ and $\alpha_2(\beta82\text{-X})_2$ where in all cases each beta chain has a single modification on either the β1 Valyl amino group or the β82 Lysyl ϵ-amino group without cross-linking. More details of the structures of the modified hemoglobins are given below. For purposes of this discussion, the results in Figure 4 show that the chromatographic positions of the modified hemoglobins found are consistent with the number and size of the dicarboxylate molecules present per hemoglobin molecule and the amino groups that have been blocked.

B. Globin Chain Products Separated By Reversed Phase HPLC:

Analytical separations of heme and globin chains were carried out by reversed phase HPLC on the reaction mixtures resulting from treating hemoglobin in different ligand states, for different concentrations of cross-linking reagents, and with different forms of the cross-linking reagents of Kluger. This reversed phase HPLC procedure was also used to follow the isolation and purification of single hemoglobin components used for further structural and functional analysis.

i. Influence of Ligand State of Hemoglobin on Reaction Products Formed: The number, kinds, and relative amounts of modified globin chains obtained by reacting hemoglobin with Kluger's reagents vary with the ligand state of the hemoglobin and with the presence or absence of 2,3-DPG as expected from the different modified hemoglobins described above. **Figure 5** shows examples of this for the reaction of FMP with deoxyHb in the absence of 2,3-DPG (Figure 5b), deoxyHb with 5 mM 2,3-DPG (Figure 5c), and COHb (Figure 5d) compared to unmodified Hb (Figure 5a). The elution times of the unmodified chains vary some between chromatograms because they were run at different times with different lots of developer; however, the relative positions are comparable. The structural identities of the modified globins are based upon studies to be described later in this report. Heme elutes at about 9 minutes. This is followed at about 23 minutes by a variable but small amount of pre-beta globin that is generally found normal hemolysates. The normal, unmodified β chains elute at about 26 minutes and the normal, unmodified α chains elute at about 32-33 minutes. Differences in the modified chains found for the three conditions are evident. The presence of DPG in deoxyHb eliminates to formation of modified β chain products. The presence of CO as the ligand alters the β chain products formed. Comparable differences between the globin chain products for CO and deoxyHb conditions have been found from similar studies of the other dicarboxylic bis (methyl phosphate) reagents of Kluger as also noted above for the studies of modified hemoglobins. Studies of the effect of DPG on the reaction of deoxyHb with IPMP show similar inhibition of the modification of β chains and enhancement of the modification of the α chains.

ii. The Effect of the Concentration of Cross-linking Reagent: **Figure 6** shows the globin chains of the hemoglobin products for three different concentrations of TPMP. As expected from the studies described earlier and in Figure 3, the extent of modifying β chains increases with concentration of reagent used. Also the extent of modifying the α chains increases significantly with increasing TPMP concentration. The complexity of the pattern of the modified chains appears to increase at higher concentrations of reagent.

iii. The Effect of Structure of the Cross-linking Reagent: **Figure 7** shows a comparison of the chromatographic behavior of the globin chains of the mixtures of modified hemoglobins resulting from reacting deoxyHb with four different dicarboxylic bis(methyl phosphate) reagents. The reaction mixtures are the same or similar to those used for the study of modified hemoglobins described above and shown in Figure 4. Variations in the elution times of globin chains as reflected in the positions of unmodified α and unmodified β chains are due to running the chromatograms at different times and with slight differences in the developer conditions. The sequences of elution of the different modified β chains are similar for the four cross-linking reagents. All elute slower than unmodified β chains. Cross-linked β chains move slower than uncross-linked but singly modified β chains. The position of elution of modified chains is also influenced by the size of the dicarboxylic acid group with the 3,3'-stilbene dicarboxylate being the slowest and fumaryl the fastest. Although modification of the α chains was not evident with the 3,3'-stilbene reagent, the other three reagents did produce one or more different modifications. The identity of the structures of many of the globin chains is based on structural characterization described below.

C. Structural Characterization Of Modified Hemoglobins By Peptide Analysis:

i. Experimental Approach: The general approach to structural characterization of the various modified hemoglobins has been to isolate single hemoglobin components by preparative chromatography. Initial separation has been achieved, in some cases, by chromatographing up to 1 gm of hemoglobin reaction mixture on a preparative anion exchange column (DEAE-Sephacel). Zones containing more than one hemoglobin component have been further separated by rechromatography on a preparative cation exchange column (CM-Sephadex C-50). In some cases preparative scale HPLC using an anion exchange column (SynChropak AX300) and/or a cation exchange column (SynChropak CM 300) has been done as the initial step or for rechromatography to further purify some hemoglobin components. The extent of purification of fractions at each step has been assessed by analytical anion exchange HPLC. In some cases it has not been possible to completely purify a modified hemoglobin; however, determination of structural modifications has still been possible by the isolation of the main modified globin chains from the partially purified hemoglobin.

The globin chains of each of the isolated hemoglobin components have been separated by both analytical and preparative reverse phase HPLC (Vydac C_4 columns) procedures. Results from the analytical HPLC analyses have been used to identify the elution position of the modified chains on the HPLC chromatograms of the original reaction mixtures. The material obtained from the preparative chain separation chromatograms has been used for SDS-PAGE studies to estimate the molecular size of the globin fraction and for further structural analyses. In some cases, preparative chain separations have been done using hemoglobin reaction mixtures in order to isolate the major modified globin components for further structural characterization.

The presence and exact nature of structural modifications of each globin chain has been determined first by hydrolyzing with trypsin sometimes followed by further hydrolysis with Glu-C endoproteinase. During the first half of the reporting period these enzyme hydrolyses were done in the absence of denaturing agents. Since then we have found that more complete hydrolysis of the modified globin chains can be obtained by doing the hydrolyses in the presence of urea as described under Material and Methods. Following hydrolysis the peptides are separated by reversed phase HPLC (Vydac C_{18} column). In the case of β chains, this procedure is generally sufficient to identify any changes in the peptide pattern. In some cases in which there is incomplete hydrolysis of a modified globin chain, the mixture of modified peptides are hydrolyzed again with the Glu-C enzyme in the presence of 0.05% SDS and then rechromatographed by reversed phase HPLC. When the initial peptide pattern reveals a modified α chain, the remaining enzyme hydrolysate is oxidized with performic acid, hydrolyzed again with trypsin in 2 M urea, and then examined by HPLC. These hydrolysis steps have generally resulted in satisfactory cleavage of the modified globin chains and permitted the isolation of all of the chemically modified peptides. The peptide patterns of each modified globin chain has been examined for the decrease or loss of any normal peptide and the appearance of new modified peptides. If no alterations are noted the chain is concluded to be unmodified. This has always correlated with normal elution by the HPLC chain separation procedure. The modified peptides have been isolated, rechromatographed, if necessary, and subjected to amino acid analysis. From the amino acid composition of the modified peptides plus the decrease or absence of one or more normal peptide, the structural modification of the globin chain can be deduced. In some cases, the results of the SDS-PAGE analysis of the purified hemoglobin component or isolated chain has been required to conclude that a cross-linking is between two globin chains rather than within one globin chain.

ii. Examples of Globin Chain Separations of Selected Modified Hemoglobins: Examples of the separation of globin chains of three modified hemoglobins are shown in **Figure 8**. Partially purified hemoglobin components were isolated from a reaction mixture resulting from treating deoxyHb with MSMP [meta or 3,3'-stilbene dicarboxyl bis(methyl phosphate)] by sequential purification first by anion exchange HPLC followed by cation exchange HPLC. All of the modified hemoglobin components shown in Figure 4d were examined by the analytical HPLC chain separation procedure. Each of these were found to have normal, unmodified α chains. Except for zone 1 of Figure 4d, the other hemoglobin components were found to have one or more chemical modifications of their β chains. The elution positions of the modified chains shown in Figure 8 have been used to identify the globin chains found in chain separation chromatograms of the original reaction mixture as illustrated in Figure 7d. Preparative scale isolation of these modified globin chains, generally from purified hemoglobin components, have been used for further structural analysis. The globin chains of the major components found in the reaction mixtures of hemoglobins modified with the other dicarboxylic bis (methyl phosphate) reagents of Kluger have also been identified in this way.

iii. <u>Examples of Peptide Patterns of Selected Modified Globin Chains:</u> The peptide patterns of the aminoethylated β chains from hemoglobins in zones 2, 5, and 6 (see Figures 4d and 8) of MSMP treated hemoglobin are shown in **Figure 9**. An ammonium acetate gradient was used for the development of these HPLC chromatograms. These show changes in the amounts of the tryptic-Glu-C peptides βT-1, βT-9, and βT-10a' (Gly-Thr-Phe-Ala-Thr-Leu-Ser-Glu) and the presence of one or more modifications of these peptides. The modified peptides found result from the reaction of the MSMP with the N-terminal amino group of valyl residue 1 of the β chain and/or the ϵ-amino group of lysyl residue 82 of the same or other β chain. When the ϵ-amino group of a lysyl residue is blocked as by a stilbene dicarboxylate group, the residue will no longer serve as a substrate for tryptic hydrolysis. The β82 lysyl residue is normally hydrolyzed by trypsin to form the βT-9 and βT-10a peptides or βT-10a' peptide in the case of additional treatment with Glu-C endoproteinase. Thus, the absence of normal βT-9 and βT-10a' from the peptide pattern of zone 6 shown at the bottom in Figure 9 indicates that the ϵ-amino group of the β82 lysyl residue has been blocked, presumably by forming an amide bond with one of the carboxyl groups of MSMP. One of the modified peptides that elutes near the end of the chromatogram was found to have the amino acid composition of βT-9 plus βT-10a' as shown in **Table I**. The other modified peptide had the same composition plus an extra lysine. The latter is due to incomplete hydrolysis of βT-8 lysine. This has subsequently been overcome by doing the enzymatic hydrolyses in the presence of urea. The strong UV absorption at 306 nm found for each of these modified peptides is also consistent with the presence of the 3,3'-stilbene group. With this structural data plus the observation that the hemoglobin in zone 6 contained only monomer chains by the SDS-PAGE procedure, it can be concluded that one of the carboxyl groups of a single stilbene dicarboxylate is attached to the β82 lysyl residue and that the other carboxyl group has not reacted with any other hemoglobin residue.

The peptide pattern of the β chain from the hemoglobin of zone 2 illustrated at the top in Figure 9 shows a reduction in the amount of βT-1, βT-9, and βT-10a' to half normal and the appearance of a new pair of peptides that elute near the end of the chromatogram. The amino acid compositions of these indicate that they both contain βT-1, βT-9, and βT-10a'. One has an extra lysine due to incomplete tryptic hydrolysis of βT-8 for reasons noted above. Both modified peptides absorb strongly at 306 nm. The hemoglobin from which these peptides were derived was found by SDS-PAGE to contain both dimers and monomers. Thus, we concluded that it has 2 normal α chains and 2 β chains cross-linked with one stilbene dicarboxylamide between the valyl β1 residue of one β chain and the lysyl $\beta 82$ residue of the other β chain. The chemical formula for this hemoglobin can be represented as $\alpha_2\beta 1$-S-82β where "S" represents a 3,3'-stilbene dicarboxylamide group.

Examination of the peptide pattern of the β chains from the hemoglobin of zone 5 shown in the middle of Figure 9 reveals no normal βT-9 or βT-10a', one half the normal amount of βT-1 and the presence of each pair of modified peptides observed for the peptide patterns for the β chains of the hemoglobins in zone 2 and zone 6. The hemoglobin of zone 5 showed cross-linked chains by SDS-PAGE. The structural modifications that can be deduced from these data are one stilbene dicarboxylamide cross-linked between the $\beta 1$ valyl residue of one chain and the $\beta 82$ lysyl residue of the other chain plus a second stilbene group attached to the $\beta 82$ lysyl residue of the first β chain. Thus there are two stilbene groups present in this modified hemoglobin. The structure can be represented as $\alpha_2\beta 82$-S-$1\beta 82$-S.

Figure 10 shows the peptide patterns of the globin chains from three different hemoglobins modified with IPMP [isophthalyl bis(methyl phosphate)] (see Figure 4b for elution positions of hemoglobins and Figure 7b for elution positions of the modified β chains). These peptides were also separated by reversed phase HPLC using an ammonium acetate gradient. The enzymatic hydrolyses were done in the presence of urea which resulted in more complete cleavage including the removal of βT-8 from the N-terminus of βT-9. The amino acid compositions of the modified peptides were determined and resulted in identifying a modified βT-9,10a' due to an IPMP group on its lysyl residue $\beta 82$ for the hemoglobin of zone 6. An identical cross-linked combination of βT-1 to βT-9,10a' was found for the major hemoglobins of both zone 5 and zone 4. From SDS-PAGE studies of these hemoglobins and their isolated chains and the determination of the relative amounts of unmodified βT-1, βT-9, and βT-10a', we can conclude that the hemoglobin in zone 2 (Figure 4b and bottom chromatogram of **Figure 10**) has two unmodified α chains and two β chains cross-linked by a single isophthalyl group between the $\beta 1$val of one chain and the $\beta 82$lys of the other chain. On the other hand, the major hemoglobin in zone 5 (Figure 4b and middle chromatogram of Figure 10) has, in addition to two normal α chains, two β chains each of which has an internal linking of the $\beta 1$val to the $\beta 82$lys of the same chain by a single isophthalyl dicarboxylamide group. The β chains are not cross-linked because only monomers were found by SDS-PAGE of this globin chain. The structure of the main hemoglobin in zone 6 (Figure 4b and top chromatogram of Figure 10) is concluded to have the structural formula $\alpha_2(\beta 82\text{-I})_2$ where "I" represents an isophthalyl group in this case on each $\beta 82$ lysyl residue. Again, SDS-PAGE showed only monomers so no cross-linking is present in this modified hemoglobin.

Peptide patterns of the modified β chains from four different hemoglobins present in zones 3 and 4 of the reaction mixture of deoxyHb treated with IPMP are shown in **Figure 11** (see

Figure 4b for elution positions of hemoglobins by anion HPLC and Figure 7 for the elution positions of the modified chains). The modified hemoglobins were isolated first by preparative anion exchange HPLC (SynChropak AX300) followed by rechromatography by preparative cation HPLC (SynChropak CM300). In one case, three different modified hemoglobins co-eluted as one hemoglobin zone designated AX4 CM2 (the third zone from the CM300 HPLC rechromatogaphy of the fourth zone from the AX300 HPLC column). Although these hemoglobins could not be resolved completely, their globin chains were resolved almost completely by reversed phase HPLC. Urea was present during the enzyme hydrolyses. Acetonitrile rather than ammonium acetate was used for the HPLC developer gradient. The amino acid compositions of the modified peptides that contained isophthalyl groups (detected by their absorption of UV light at 257.5 nm) are shown in **Table II.** From these data, the relative amounts of βT-1, βT-9, and βT-10a' in the peptide patterns, and the behavior of the isolated chains on SDS-PAGE, the structures of each of the globin chains and the hemoglobins from which they were derived were deduced. The peptide pattern shown in Figure 11a, for the globin designated IPMP AX3 CM3 II, results from β chains cross-linked by a single IPMP between the β82lys of one and the β82lys of the other. The formula of the hemoglobin would be $\alpha_2(\beta 82\text{-I-}82\beta)$. This was found to be one of the two major modified hemoglobins when COHb was reacted with IPMP. The second peptide pattern shown in Figure 11b, designated IPMP AX4 CM2 IV, is concluded to be from a β dimer cross-linked by one isophthalyl group between the β1val of one chain and the β82lys of the second chain which also has a second isophthalyl group on its β1val. The small amount of normal βT-1 that was detected is believed to be a contaminant from another modified globin that was not completely resolved by the preparative chain separation procedure. The third globin, designated IPMP AX4 CM2 V (peptide pattern shown in Figure 11c), is concluded to have a formula of β82-I-1β82-I, i.e., a β dimer cross-linked by one isophthalyl group between the β82lys of one chain and the β1val of the second chain which also has a second IPMP group on its β82lys. The globin chain for the peptide pattern in Figure 11d, designated IPMP AX4 CM2 VI, has two cross-linkages with one isophthalyl group between the β1val of one chain and the β82lys of the other and a second isophthalyl group between the β1val of the second chain and the β82lys of the first chain. The peptide pattern and amino acid composition of the modified peptide of this doubly cross-linked globin are identical to those of the β chain that is internally linked between the β1val and the β82lys of the same chain. The peptide pattern but not the modified peptide differs from that of the singly cross-linked globin with the formula β1-I-82β described above (Figure 10c). The singly cross-linked globin chains show the presence of half the normal amounts of βT-1, βT-9 and βT-10a' rather than the complete absence of these normal tryptic peptides.

iv. Structures of Globin Chains Found in Hemoglobins Treated with Four Different Cross-linking Reagents from Professor Kluger: The structures of the globin chains of the major and some minor hemoglobins that have been isolated from the reaction of hemoglobins in various ligand conditions with fumaryl bis(methyl phosphate), FMP, are listed in **Table III.** The zones of the hemoglobins components that were isolated from the SynChropak AX300 anion exchange HPLC column are designated by "AX" numbers in sequence of their elution. Further purification was then done by rechromatography of the AX zones on a preparative size SynChropak CM300 cation exchange HPLC column. Zones from the cation column, designated by "CM" numbers, were then subjected to globin chain separation using a preparative Vydac C_4 large pore reversed phase HPLC column. The elution position of the globin chains are represented by Roman numeral. As noted earlier, the structures of the major hemoglobins have been designated on the hemoglobin chromatogram for the deoxyHb reaction mixture shown in Figure 4a. The globin structures have also been shown on Figure 7a. In addition to unmodified hemoglobin the major modified hemoglobins found for the reaction of FMP with deoxyHb (in the absence of DPG) were $\alpha_2(\beta82\text{-F})_2$, $\alpha_2(\beta_{82}{}^1\text{>F})_2$, and $\alpha_2\beta1\text{-F-}82\beta$. The reaction of FMP with deoxyHb in the presence of 2,3-DPG does not produce any modified β chains but does produce $\alpha99\text{-F-}99\alpha\beta_2$ and $(\alpha1\text{-F})_2\beta_2$ as major modified products. The major modified hemoglobin produced in the reaction of FMP with COHb is $\alpha_2\beta82\text{-F-}82\beta$. Not shown in Table III but based on chromatographic characterization, another modified hemoglobin formed with COHb is $\alpha_2(\beta82\text{-F})_2$. This is also found in the case of reacting FMP with deoxyHb.

The structures of the hemoglobins isolated from the reaction of hemoglobin in the deoxy and CO forms with isophthalyl bis(methyl phosphate) (IPMP) are listed in **Table IV.** The structures of the major hemoglobins have been designated on the hemoglobin chromatogram for the deoxyHb reaction mixture shown in Figure 4b. The globin structures have also been shown on Figure 7b. In addition to unmodified hemoglobin the major modified hemoglobins from the reaction of IPMP with deoxyHb (in the absence of DPG) are $\alpha_2(\beta82\text{-I})_2$, $\alpha_2(\beta_{82}{}^1\text{>I})_2$, and $\alpha_2\beta1\text{-I-}82\beta$. Smaller amounts of the following hemoglobins were also found: $\alpha_2(\beta82\text{-I})_2$, $\alpha_2\beta82\text{-I-}82\beta$, $\alpha_2\beta82\text{-I-}1\beta82\text{-I}$, $\alpha_2\beta1\text{-I-}82\beta$double cross-linked, $\alpha99\text{-I-}99\alpha\beta_2$, and $\alpha99\text{-I-}139\alpha\beta_2$. The major modified hemoglobin formed by treatment of COHb with IPMP was found to be $\alpha_2\beta82\text{-I-}82\beta$. Some $\alpha2(\beta82\text{-I})_2$ has also been identified by comparison of the chromatographic behavior of the modified hemoglobins of the COHb and deoxyHb reaction mixtures.

The structural characterization of the globin chains of modified hemoglobins isolated from the reaction mixture resulting from treating deoxyHb with terephthalyl bis(methyl phosphate) (TPMP) is still in progress. The probable structures of the modified globin chains based upon the peptide patterns and comparisons made to the IPMP products are shown in Figure 7c. It appears that most of the modifications observed for IPMP are also present in the reaction mixture of deoxyHb treated with TPNP.

The structures of the globins isolated from the major hemoglobin components that can be separated from the reaction mixture resulting from treating deoxyHb with meta or 3,3'-stilbene dicarboxyl bis(methyl phosphate) (MSMP) are shown in **Table V.** They are also indicated on chromatograms of Figures 4d and 7d. Also summarized in Table V are the findings from SDS-PAGE, globin chain separation, and peptide patterns. Fewer modified hemoglobins and globin chains have been found in the reaction of MSMP with deoxyHb than for the other three reagents of Kluger that have been described above. The major MSMP hemoglobins that have been characterized are: $\alpha_2\beta$1-S-82β, $\alpha_2\beta$82-S-82β, $\alpha_2\beta$82-S-1β82-S, and $\alpha_2(\beta82\text{-S})_2$. A β chain internally linked from β1val to β82lys of the same chain has not been observed for the MSMP reacted hemoglobin. Other minor components that appear to be present based upon the appearance of the anion exchange separation shown in Figure 4d have not yet been isolated and studied.

v. <u>Comparison of Modified Globin Chains Obtained with Dicarboxylic Bis(Methyl Phosphate) Reagents of Kluger:</u> A summary comparison of the structures of the modified globins found in the reaction mixtures of hemoglobins treated with three of the dicarboxylic bis(methyl phosphate) reagents of Kluger is shown in **Table VI.** Structures of the terephthalyl modified hemoglobins are not included because the studies are not yet complete. It would appear from this table that α chains are not accessible to the stilbene reagent but are to the fumaryl and isophthalyl, especially when the central cavity between the β chains is occupied by DPG. The β82lys residue appears to be available to react with all of the reagents when the hemoglobin is in either the low affinity state (deoxyHb) or high affinity state (COHb). On the other hand, the β1Val residue appears to react with these reagents only when the hemoglobin is in the low affinity state in the absence of DPG. This residue does not react with any of the reagents when the hemoglobin is in the high affinity state.

One of the major modified hemoglobins formed under deoxy conditions with the fumaryl and isophthalyl but not the stilbene dicarboxyl reagent has intra-chain linkage between the β1val and β82lys residues of the same chain. This derivative has also been observed for the terephthalyl reagent. All of Kluger's reagents so far tested will form modified hemoglobins with cross-linking between the β1val residue of one chain and the β82lys of the other chain when the reaction is carried out with deoxyHb. This product is not formed with COHb. Additional modifications of the β1-X-82β cross-linked β chains have been demonstrated, at least for the isophthalyl and stilbene dicarboxyl reagents. Both of these reagents form small amounts of this cross-linked product with an additional group on the second β82lys residue. In the case of the isophthalyl reaction mixture, a doubly cross-linked modification has been found. Small amounts of an uncross-linked product is formed with fumaryl and isophthalyl on the β1val residue. More of the β82lys modified but uncross-linked product is formed especially with the isophthalyl and stilbene dicarboxyl reagents.

The main hemoglobins found for the reaction of COHb with all of Kluger's reagents are those with the β82 residue modified, either with a single reagent group on each β82lys or one group cross-linking between the β82 residues of the two chains.

Small amounts of α99lys cross-linked hemoglobins are formed in the reaction of the fumaryl and isophthalyl reagents with deoxyHb. In the case of the isophthalyl reaction a small amount of what appears to be α99-I-139α has been found. The yields of these modified hemoglobins increase significantly when DPG is present during the reaction with deoxyHb. Some α1val modified but uncross-linked hemoglobin is formed in the reaction of the fumaryl reagent with deoxyHb. This increases substantially with DPG present during the reaction.

No cross-linking between the two N-terminal valyl residues, i.e. β1-X-1β, has been found for the reaction of hemoglobin in any ligand state with any of Kluger's reagents.

D. Specificity and Sequence of Reaction of Dicarboxylic Bis(Methyl Phosphate) Reagents with Hemoglobin:

The structural studies of the reaction products of hemoglobin treated with the dicarboxylic bis(methyl phosphate) reagents of Professor Kluger indicate that these reagents are quite specific, at least under the conditions used. Of all of the possible amino groups that might react, only those of β1val, β82lys, α1val, α99lys, and probably α139lys do react. As discussed above, the reactivity of these residues is influenced by the ligand state of the hemoglobin, presence of 2,3-DPG, and the size of the bridging group in the reagent. The degree of specificity of reaction of these bifunctional reagents for the β chain is even greater than the high specificity of the monofunctional methyl acetyl phosphate reported by Ueno et al (52). The latter reagent also reacts with β144lys.

In an attempt to explain the types of cross-linked products formed, the distances between the various reactive residues have been examined and are listed in **Table VIIA**. These distances were obtained by Dr. Daniel Tzu-bi Shih examining the X-ray crystallographic data obtained for hemoglobin (52, 53) with an interactive macromolecular graphics computer program for personal computers(Promodeler I from New England Biographics, Peacham, VT). Distances are shown for both the deoxy and oxy conformations of human hemoglobin. The distances that could be spanned by each of several cross-linker reagents are also given in **Table VIIB**. The measurements are between nitrogen atoms assuming each reagent has reacted with two different amino groups. The bridging distances for the fumaryl, isophthalyl, and terephthalyl reagents appear to be too short to form cross-links between β1val of one chain and the β82lys of the other or between the two β82lys in either the oxy or deoxy conformation. Assuming these distances for the hemoglobin residues are correct, there must be some movement of the hemoglobin molecule under the reaction conditions because these amino groups are bridged by these reagents. From the earlier work of Kavanaugh et al (21,x1) whc showed cross-linking between the two β1 valyl residues with DIDS, which has a span of about 16 Å, it can be concluded that the distances between some of the amino groups must be less than those shown in Table VII, at least for some of the time. These distances may change with the presence of the cross-linking reagents in the central cavity between the two β chains.

From the relative amounts of the various hemoglobin products formed it can be concluded that the ϵ-amino group of β82lys reacts more readily with each of Kluger's reagents than does the α-amino group of the β1 valyl residue. Once a molecule of one of the dicarboxylic bis(methyl phosphate) reagents reacts with a β82lys residue, the other acyl methyl phosphate group appears to react with either the β1val or the β82lys of the other chain or, in the case of the smaller bridging groups, with the β1val of the same chain. The fact that appreciable amounts of β82-X are formed with each of Kluger's reagents reacting with either deoxyHb or COHb may be the result of hydrolysis of the second methyl phosphate group from the reagent before cross-linking can occur. As noted before, reactions with the β1val amino group occur only when the hemoglobin is in the deoxy conformation.

E. Hemoglobin-Oxygen Equilibrium Studies of Modified Hemoglobins:

The functional properties of purified fractions of hemoglobins modified with different dicarboxylic bis(methyl phosphate) reagents of Kluger are currently being studied by measuring their oxygen equilibrium properties. A standard condition of pH 7.4 in 50 mM bis-Tris, 0.1 M Cl^-, 25° C, 55 μM heme has been used for initial comparisons. The P_{50} and in most cases the n_{50} values for the modified hemoglobins that have been studied to date are shown in **Table VIII**. Except the $\alpha_2\beta$1-S-82β hemoglobin produced with the 3,3'-stilbene dicarboxyl bis(methyl phosphate), all of the other modified hemoglobins studied to date have decreased affinities for oxygen compared to unmodified HbA. The most significant and potentially useful changes are found for the singly and doubly cross-linked hemoglobins with isophthalyl bridging between β1val and β82lys. It is estimated that the P_{50}'s for these two isophthalyl modified hemoglobins would be at or above 27 torr at pH 7.4 and 37° C. Further studies of the functional properties are in progress and will be presented in the final report.

F. Work in Progress and Plans for Future Studies:

Work in progress includes the structural characterization of modified hemoglobins produced by the reaction of deoxyHb and COHb with the terephthalyl bis(methyl phosphate) reagent of Kluger. The preliminary screening of the functional properties of the modified hemoglobins produced with the fumaryl, isophthalyl, and 3,3'-stilbene reagents is also in progress. These will be completed before deciding how much to do with the functional characterization of the terephthalyl modified hemoglobins.

A collaborative study is currently under way with Dr. Richard Brennan to isolate, crystallize, and determine by X-ray crystallography the structure of the isophthalyl and 3,3'-stilbene cross-linked hemoglobins. Although some crystals have been produced, X-ray data have not yet been collected.

During the first part of May 1990, studies were initiated with a new reagent, 1,3,5-benzene tricarboxylic tris(methyl phosphate) prepared by Ms. Jolanta Wodzinska, a graduate student of Professor Kluger. Structural studies of the products formed with deoxyHb and COHb are in progress in collaboration with Ms. Wodzinska and Professor Kluger.

The effect of temperature on the reaction of Kluger's reagents with hemoglobin are planned in order to determine its effect on the relative yield of the reaction products.

Although results from studies of the modification of hemoglobin with Kluger's reagents obtained to date are promising, effort will be directed to designing new cross-linkers that will react more specifically and in higher yields. Attempts will be made to design and test reagents that will cross-link the two β1val residues. Reagents with more negative charges will be sought with the expectation that they should bind more strongly to the DPG binding site.

PART II: CHROMATOGRAPHIC AND STRUCTURAL CHARACTERIZATION OF HEMOGLOBINS IN DBBF-HB FROM BAXTER TRAVENOL:

A. Characterization of the Main Hemoglobin Component in the DBBF-Hb Preparation:

i. Separation of Hemoglobins by Ion Exchange HPLC: A sample of DBBF-Hb (hemoglobin cross-linked by reacting deoxyHb in the presence of 2,3-DPG with 3,5-dibromosalicyl-bis fumarate) was obtained from Dr. Mario Marini of LAIR. It was from Batch 11 produced by Baxter Healthcare Corp. (Round Lake, IL) under contract from the United States Army Medical Research and Development Command. The purity of the sample was first studied by analytical ion exchange HPLC. **Figure 12** shows the chromatographic separation using the PolyCAT A poly(aspartic acid) silica cation exchange HPLC column. This indicates that the sample is heterogeneous with one single major hemoglobin component comprising about 45% of the total. The remaining hemoglobin was poorly resolved into 14 or more minor components one of which eluted in the position of unmodified Hb A. It amounted to about 5% of the total hemoglobin. **Figure 13** shows the anion exchange HPLC chromatogram of the sample using an analytical SynChropak AX300 column. About 50% of the DBBF-Hb eluted in this system as a single major component. Again, a peak representing about 5% of the total eluted in the position of unmodified Hb A. Another 8 or more poorly resolved minor components were observed.

ii. Separation of Globin Chains by Reversed Phase HPLC: **Figure 14** is an analytical globin chain separation of a sample of the DBBF-Hb Batch 11 using a large pore C_4 reversed phase HPLC column. It shows two major globin chains and several minor products. The first major globin chain component elutes in the position of normal, unmodified β chains. The other major component elutes very late and, as will be shown below, is the α99-F-99α cross-linked derivative. One of the minor components elutes in the position of normal, unmodified α chains and is presumably due to unmodified Hb A present in the original Batch 11 DBBF-Hb. There are at least 7 other minor globin chain components that taken together appear to account for about 10-15% of the total globin chains, or 20 to 30% of the non-α chains.

It is concluded from this chain separation, the ion exchange chromatography of the hemoglobins, and structural characterization of the major modified α chain that the major modified hemoglobin component represents about half of the total hemoglobin in the Batch 11 DBBF-Hb. Unmodified hemoglobin accounts for about 5% of the total.

iii. Structural Characterization of the Major Modified Globin: The major modified globin component in the Batch 11 DBBF-Hb was isolated by preparative HPLC chain separation from the whole sample that had not been purified further. This globin was subjected to structural analysis by tryptic hydrolysis, reversed phase HPLC peptide separations, and quantitative amino acid analysis. Because of the presence of a cysteinyl residue at position 104 of the α chain, the globin was either oxidized with performic acid or aminoethylated to prevent formation of disulfide bonds. In some cases urea was added during enzyme hydrolysis to obtain more complete cleavage of resistant bonds.

Figure 15 is the tryptic peptide pattern of oxidized, modified globin hydrolyzed with trypsin only and without urea. It reveals all of the normal peptides of unmodified α chains except for the absence of αT-11 and αT-12 and a reduction in the amount of αT-13. In addition, there is a complex of three new peaks that elute near the end of the chromatogram. Quantitative amino acid analysis of these new peptides indicated that the first contains equal molar amounts of αT-11 and αT-12, the second contains two moles of αT-11 and αT-12 each plus one mole of αT-13, and the third contains equal molar amounts of all three of these tryptic peptides. As shown in **Figure 16**, much greater hydrolysis of the αT-13 tryptic peptide from the modified αT-11,12 was achieved by hydrolyzing with trypsin in the presence of urea.

In order to be able to account for all of the normal tryptic peptides of the α chain an aminoethylated preparation of the major modified globin from the DBBF-Hb sample was also hydrolyzed with trypsin in the presence of urea. The peptide pattern is shown in **Figure 17**. The main modified tryptic peptide contains two moles of αT-11 and αT-12 to one mole of αT-13. This peptide must result from the failure of tryptic hydrolysis of both α99lys residues and one of the α127lys residues. The resistance of this one α127lys residue to tryptic hydrolysis of this aminoethylated preparation is presumably due to the retention of some secondary or tertiary structure in the cross-linked globin that does not permit access of trypsin to an otherwise susceptible bond. The observation of almost complete hydrolysis of this bond in the case of the oxidized globin treated with trypsin in the presence of urea indicates that neither α127lys bond is blocked by fumarate. On the other hand, the complete resistance of the α99lys bond to hydrolysis by trypsin is consistent with it being blocked by fumarate. This peptide data plus the finding of dimer chains by the SDS-PAGE procedure indicate that this major modified globin is cross-linked between the α99lys residues of two α chains. This is consistent with the observation published by Chatterjee et al (27) that fumarate forms a diamide linkage between the ϵ-amino side chains of the two α99 lysyl residues. No other modifications of the major modified globin were detected.

B. Studies of the Minor Hemoglobin Components in the DBBF-Hb Preparation:

The nature of the minor components in Batch 11 DBBF-Hb has been studied further by isolating several of them by preparative ion exchange HPLC chromatography. The mixture was first separated with a preparative SynChropak AX300 column followed by rechromatography with a Synchropak CM300 column. Globin chains were then isolated by reversed phase HPLC chromatography from some of these purified hemoglobin fractions. Peptide patterns of hydrolysate of some of these chains were studied by reversed phase HPLC using a C_{18} column in order to detect structural modification. In **Table IX** are listed the various components and approximate percentages that were obtained. The following observations and conclusions can be made:

1.) Except for a zone containing only unmodified hemoglobin, all of the other minor hemoglobin components that were isolated and studied appear to have α chains cross-linked between the two α99lys residues. No hemoglobin component other that the minor component of unmodified Hb A appeared to contain unmodified α chain. **Figure 18** shows the chain separation for several of the hemoglobin fractions that were isolated.

2.) Most of the minor hemoglobin components studied have β chains that elute like normal, unmodified β chains on reversed phase HPLC. Fractions AXI, AXIICMb, AXIICMc, AXIII CMb, and AXIV show the major or sole non-α chain fraction elutes in the position of the unmodified β chain. This is also evident from Figure 18. In the case of hemoglobin fractions AXIICMa, AXIIICMa, and AXV, most or all of β chains elute either ahead or behind the position of normal β chain.

3.) The peptide patterns of all modified α chain fractions studied had the characteristic peak eluting at about 94 minutes that represents the α99lys cross-linked peptide. Four of these fractions were analyzed as shown in **Figure 19**.

4.) Peptide patterns of 5 of the β chains isolated from the major and 4 different minor hemoglobin fractions were studied. These are shown in **Figure 20**. The pattern for the β chain of AXI is identical to normal, unmodified β chain. Similar patterns were obtained for the normally eluting β chains for hemoglobin fractions AXIIICMb, and AXIVCMmaj. On the other hand, the patterns for the two modified β chains obtained from AXVCMmaj do not show the normal βT-10,11 or βT-10,11,12 peptides. There is also a new peak eluting just behind βT-2. Possible conclusions are that either one or both of the cysteine residues (β93 or β112) have been modified or a lysyl group in βT-10,11,12 has been modified in such a way that the splitting of β104Arg between βT-11 and β-12 is inhibited even more than in the normal. In addition, the modified βT-10,11,12 peptide probably elutes with the column wash at the end of the chromatogram. The nature of the peptide eluting after βT-2 is unknown. Because of the small amount of material isolated we have not carried the structural characterization of these fractions further.

The estimates of the relative amounts of the minor hemoglobin components isolated and studied as described above are only approximate. However, the data indicate that at least 90 but not more than 95% of the α chains in the hemoglobin mixture in Batch 11 DBBF-Hb is α99-F-99α cross-linked. This appears to be the only kind of α chain in all of the fractions except for the minor component of unmodified hemoglobin that represents about 5% of the total. The relative amount of β chain that appears to be unmodified as judged by elution position on chain separation and by peptide pattern analysis appears to be at least 70% but probably not more than 80%. Although data from the chain separations and peptide patterns indicate that the rest of the β chains are chemically altered, we do not know specifically how they are modified. Some of the altered β chains elute from the chain separation column at volumes observed for several of the fumaryl modified β chains that have been found for hemoglobin reacted with the fumaryl bis(methyl phosphate) reagent of Kluger. These include the β1-F, β82-F, β_{82} >F, and β1-F-82β. However, peptide patterns of these modified globins have not been obtained to prove these structures in the case of the DBBF-Hb preparation.

In summary, about 90-95% of the hemoglobin in Batch 11 DBBF-Hb is α99-F-99α cross-linked. Fifty percent of the total hemoglobin chromatographs as a single major hemoglobin component and contains β chains that appear to have chromatographic properties and the peptide pattern of normal, unmodified β chains. The remaining hemoglobin is made up of about 5% unreacted Hb A, and 45% of a mixture of minor hemoglobins that chromatograph differently than either unmodified Hb A or the major cross-linked component. However, the β chains of about half of these minor hemoglobins elute from the chain separation column in the position of normal, unmodified β chains. Some of these β chains have normal peptide patterns. The rest of the minor components have β chains that elute abnormally. Some of these β chains have altered peptide patterns. The reasons for the unusual chromatographic properties of the β chains of about half of the minor components have not been determined yet.

4. CONCLUSIONS

A. Modification of Hemoglobin with Dicarboxylic Bis(Methyl Phosphate) Reagents:

The dicarboxylic bis(methyl phosphate) reagents of Professor Kluger react readily with human hemoglobin to form a mixture of modified hemoglobins. These reagents react only with the amino groups of β1val and β82lys residues of the beta chain and mainly with the α99lys and to lesser extent with α1val and α139 residues of the alpha chain. The extent of reaction with each of these amino groups depends upon the ligand state of the hemoglobin, the presence or absence of 2,3-DPG, and the nature of the bridging group of the cross-linking reagent. In all cases so far tested, two or more hemoglobin products are obtained for each reagent and for each reaction condition. Appreciable amounts of uncross-linked hemoglobins are formed. This is partly due to internal linking between two different amino groups on the same chain and partly due to failure of the second acyl methyl phosphate to react, possibly because of competing hydrolysis reactions. Cross-linking between β1val and β82lys is obtained only by reactions with deoxyHb. Cross-linking between the two β82lys residues is obtain to a greater extent from reactions with COHb than with deoxyHb.

Cross-linking between the two α99lys residues is obtained with the smaller reagents reacting with deoxyHb especially when 2,3-DPG is present. Although the fumaryl bis(methyl phosphate) reagent of Kluger (FMP) might be used as an alternate to 3,5-dibromosalicyl-bis fumarate (DBBF) for obtaining α99-F-99α cross-linked hemoglobin, preliminary results failed to demonstrate significantly better yields of the cross-linked product when using FMP.

Incomplete functional studies of some of the hemoglobins cross-linked with Kluger's reagents show decreases in oxygen affinity especially in the case of the isophthalyl derivative, $\alpha_2\beta$1-F-82β. This cross-linked hemoglobin has an oxygen affinity that is in the physiologically useful range. However, it represents no more than 20% of the total modified hemoglobin produced under the reaction conditions tested. Further measurement of the functional properties of other derivatives and attempts to improve the reaction conditions are planned.

Cross-linking of the two β N-terminal valyl amino groups has not been obtained with any of Kluger's reagents. Although the stilbene dicarboxyl bis(methyl phosphate) reagents were synthesized as analogues to DIDS, they failed to form any β1-X-1β cross-linked hemoglobin as does DIDS. This is probably due to the fact that amide-N to amide-N spans that can be obtained by the Kluger reagents are less than that for DIDS. Attempts will be made to synthesize and test reagents with longer cross-linking spans and greater specificity.

<u>B. Chromatographic and Structural Characterization of Baxter Batch 11 DBBF-Hemoglobin:</u>

Chromatographic and structural characterization of the hemoglobins present in Batch 11 DBBF-Hb from Baxter Travenol indicate that it is comprised of about 50% of a single major component and 10 or more minor components. The major component has the molecular formula of α99-F-99$\alpha\beta_2$. Its only modification is a fumaryl cross-link between the ϵ-amino groups of the two α99lys residues. The minor components range from less than 5% to about 15%. Except for a small amount of unmodified HbA (5% of total) which contains unmodified α chains the other minor components all appear to contain the α99-F-99α modification. Although the β chains of some of these minor components are chemically altered, the β chains of others have normal chromatographic and peptide patterns. Explanations for the unusual chromatographic behavior of these minor hemoglobin that appear to have the same peptide structure as the major modified hemoglobin component is not clear. Some changes in three dimensional structure may have occurred in the heat inactivation of virus. There may be oxidation of cysteinyl and/or methionyl residues. If more structural studies are to be pursued, it would be important to examine several different batches by ion exchange chromatography first to determine the variability of hemoglobin heterogeneity.

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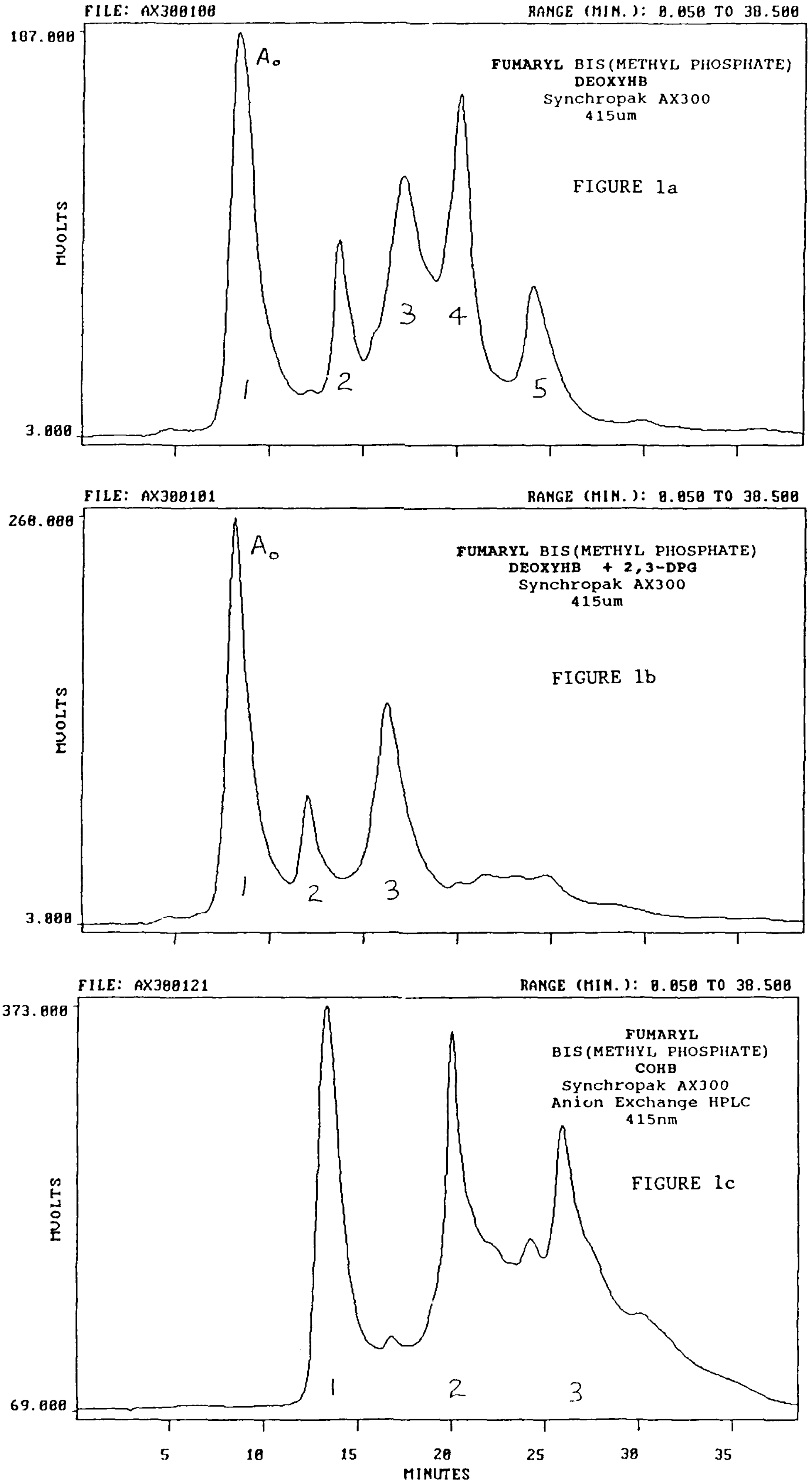
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RANGE (MIN.): 0.050 TO 38.500
187.000
3.000
MVOLTS
FUMARYL BIS(METHYL PHOSPHATE)
DEOXYHB
Synchropak AX300
415um
FIGURE 1a
A_o
1
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3
4
5
FILE: AX300101
RANGE (MIN.): 0.050 TO 38.500
260.000
3.000
MVOLTS
FUMARYL BIS(METHYL PHOSPHATE)
DEOXYHB + 2,3-DPG
Synchropak AX300
415um
FIGURE 1b
A_o
1
2
3
FILE: AX300121
RANGE (MIN.): 0.050 TO 38.500
373.000
69.000
MVOLTS
FUMARYL
BIS(METHYL PHOSPHATE)
COHB
Synchropak AX300
Anion Exchange HPLC
415nm
FIGURE 1c
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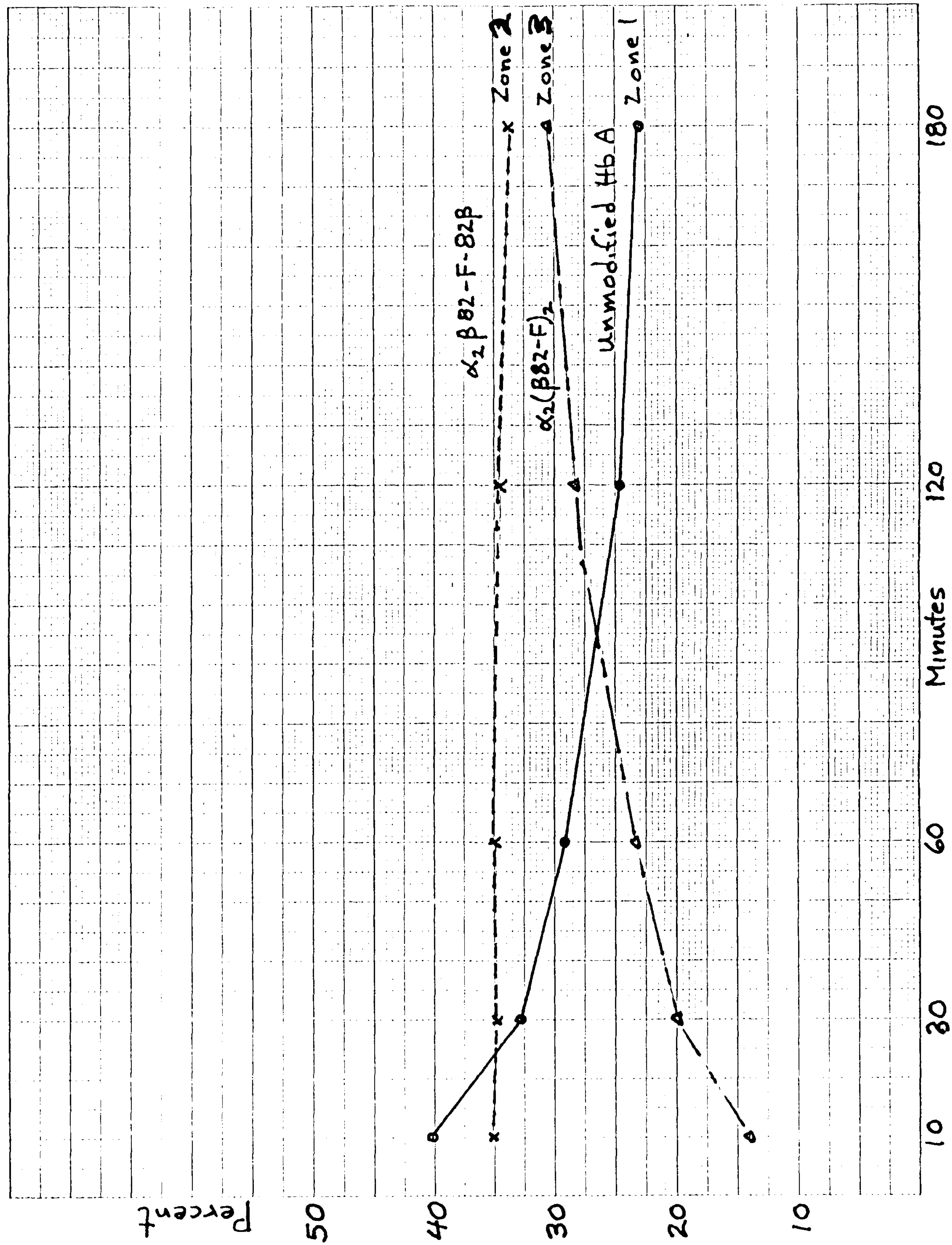
MINUTES

FIGURE 1



Zone 2
Zone 3
Zone 1
$\alpha_2\beta^{82}$-F-82β
$\alpha_2(\beta 82\text{-F})_2$
Unmodified Hb A
Minutes
10
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120
180
Percent
50
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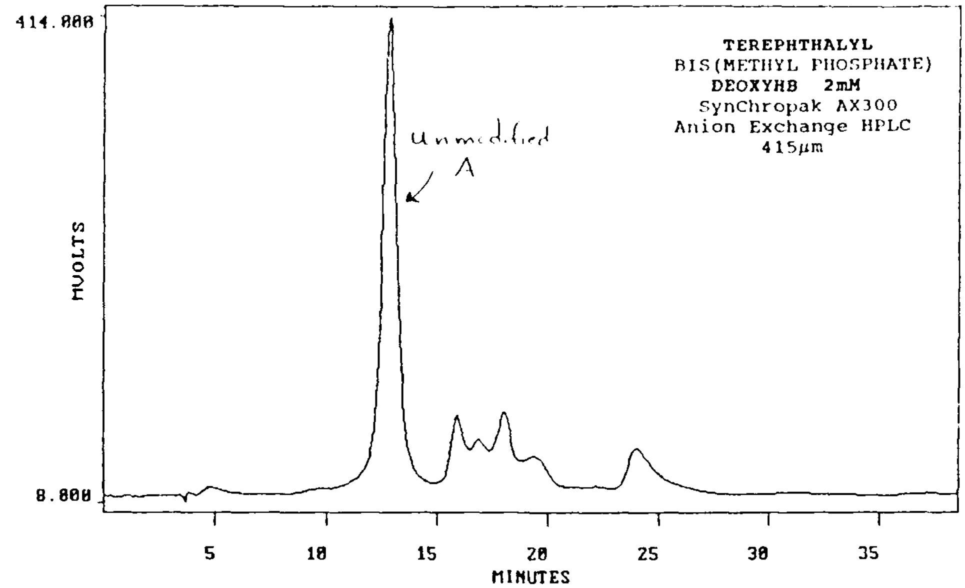
20
10

FIGURE 2



414.000
8.000
MVOLTS
MINUTES
5
10
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BIS(METHYL PHOSPHATE)
DEOXYHB 2mM
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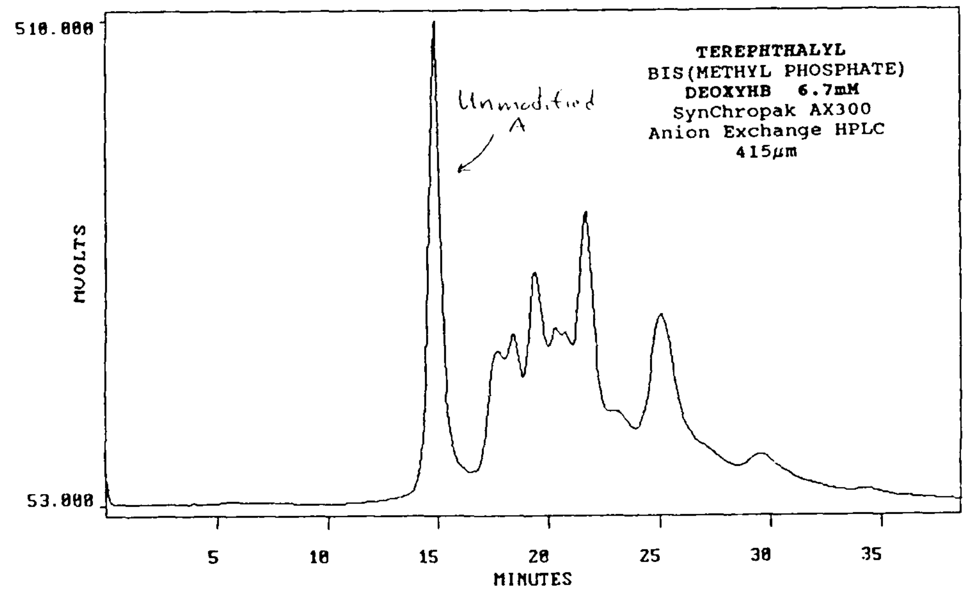
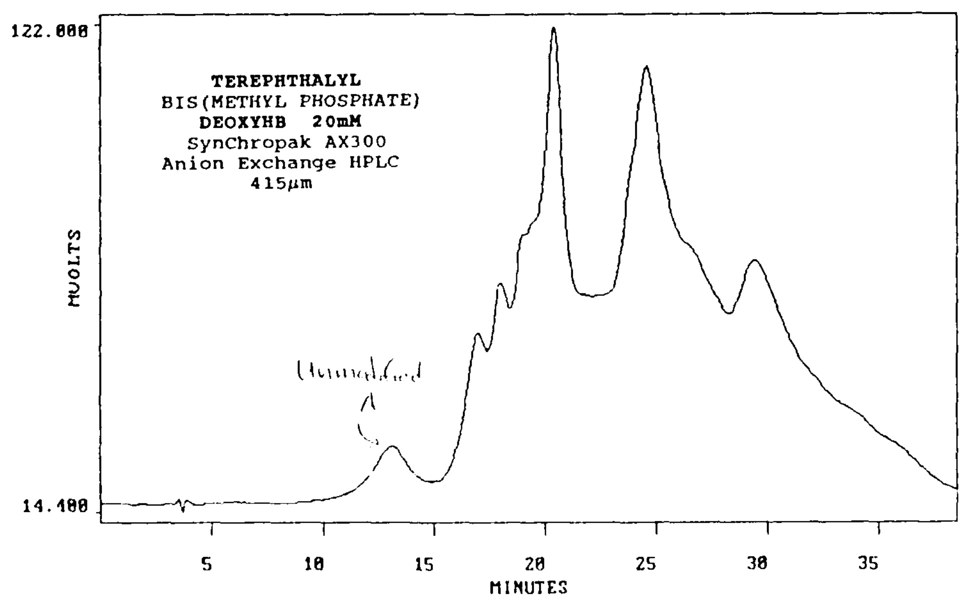
Unmodified A



510.000
53.000
MVOLTS
MINUTES
5
10
15
20
25
30
35
TEREPHTHALYL
BIS(METHYL PHOSPHATE)
DEOXYHB 6.7mM
SynChropak AX300
Anion Exchange HPLC
415μm
Unmodified A



122.000
14.400
MVOLTS
MINUTES
5
10
15
20
25
30
35
TEREPHTHALYL
BIS(METHYL PHOSPHATE)
DEOXYHB 20mM
SynChropak AX300
Anion Exchange HPLC
415μm
Unmodified A

FIGURE 3

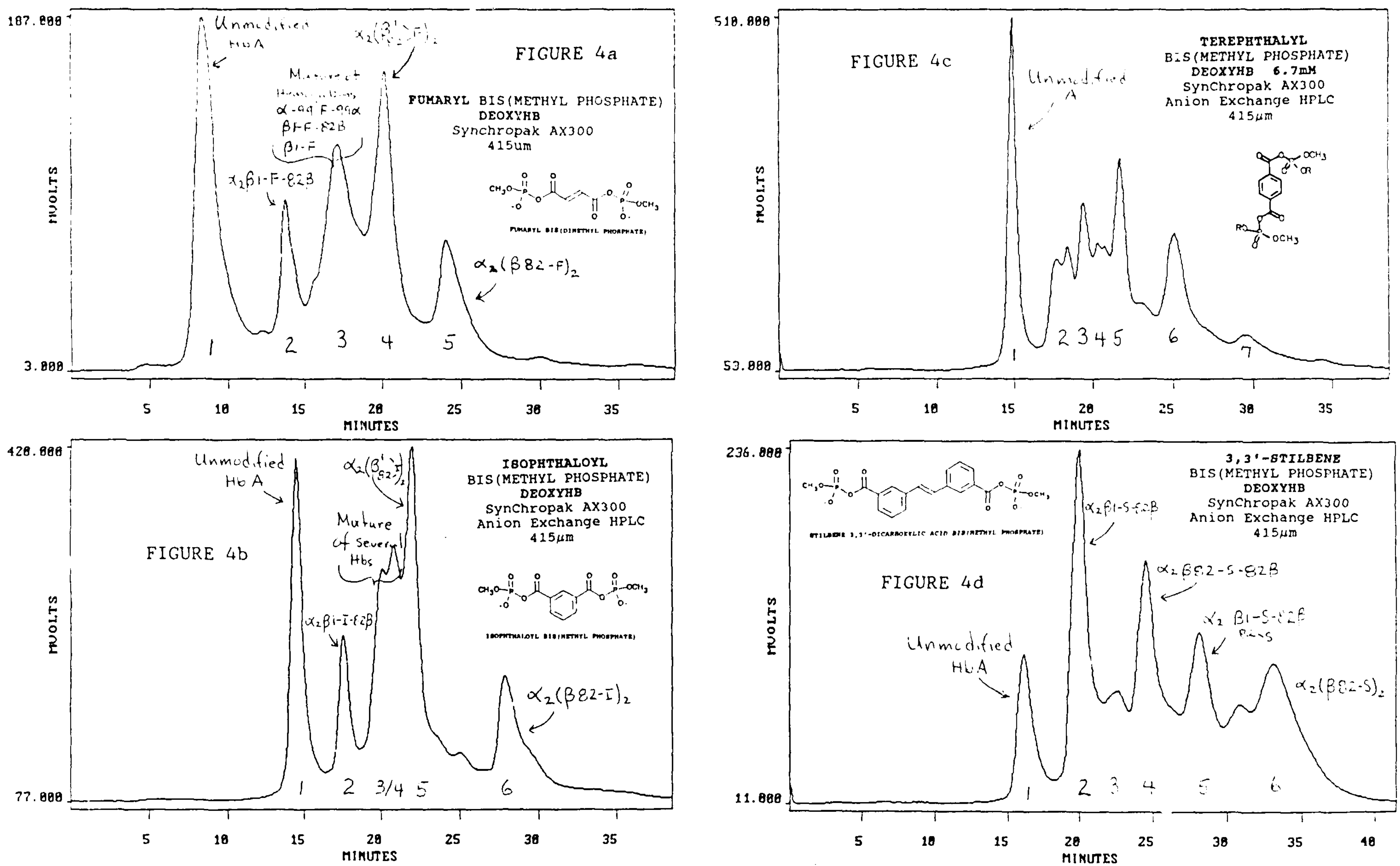
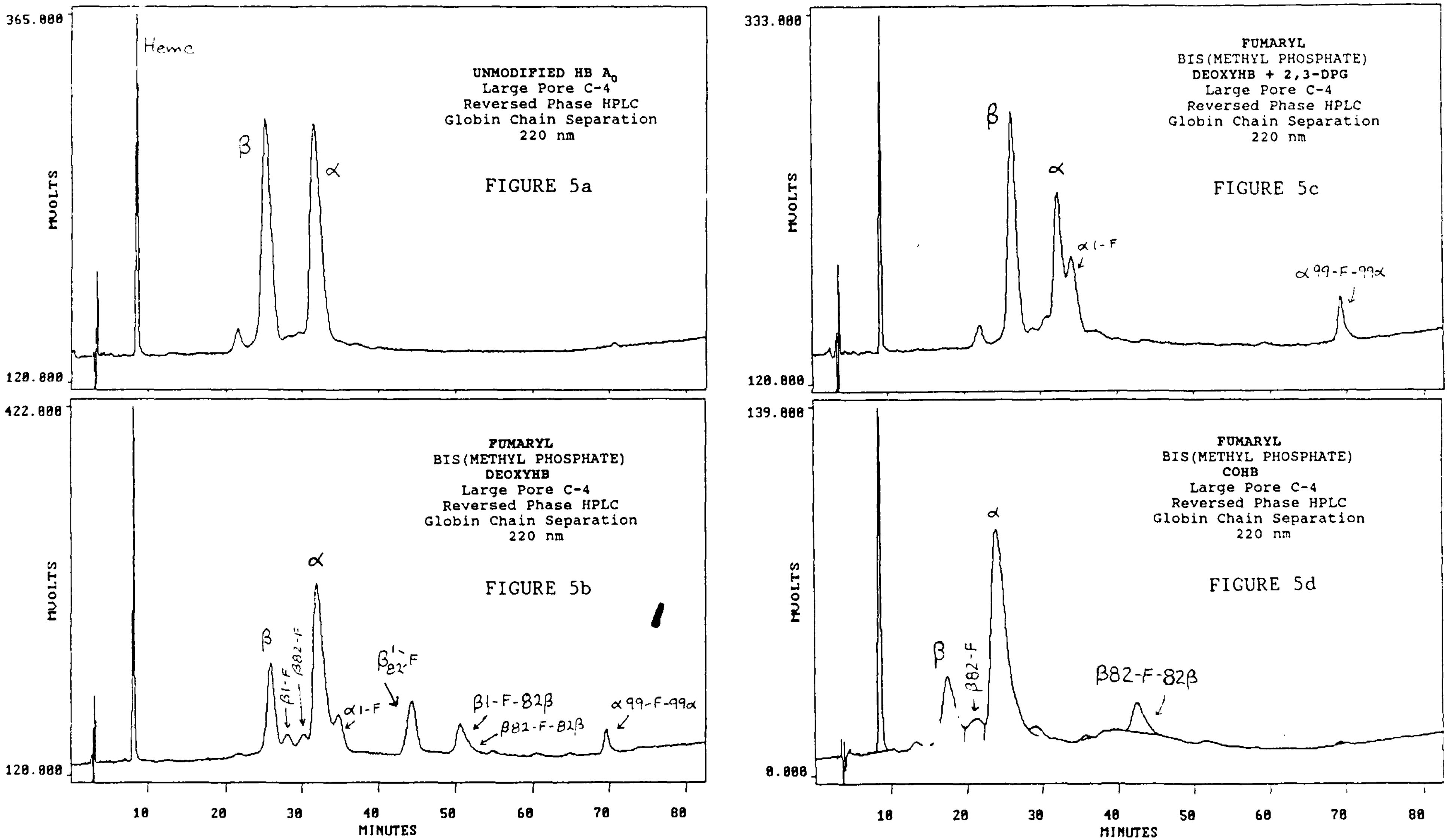


FIGURE 4a
FUMARYL BIS(METHYL PHOSPHATE)
DEOXYHB
Synchropak AX300
415um
FUMARYL BIS(DIMETHYL PHOSPHATE)
Unmodified HbA
$\alpha_2\beta 1$-F-82B
$\alpha_2(\beta 82$-F$)_2$
MVOLTS
MINUTES
FIGURE 4b
ISOPHTHALOYL
BIS(METHYL PHOSPHATE)
DEOXYHB
SynChropak AX300
Anion Exchange HPLC
415µm
ISOPHTHALOYL BIS(METHYL PHOSPHATE)
Unmodified Hb A
Mixture of Several Hbs
$\alpha_2\beta 1$-I-82B
$\alpha_2(\beta 82$-I$)_2$
FIGURE 4c
TEREPHTHALYL
BIS(METHYL PHOSPHATE)
DEOXYHB 6.7mM
SynChropak AX300
Anion Exchange HPLC
415µm
Unmodified A
FIGURE 4d
3,3'-STILBENE
BIS(METHYL PHOSPHATE)
DEOXYHB
SynChropak AX300
Anion Exchange HPLC
415µm
Unmodified HbA
$\alpha_2\beta 1$-S-82B
$\alpha_2\beta 82$-S-82B
$\alpha_2(\beta 82$-S$)_2$

FIGURE 4



365.000
120.000
MVOLTS
Heme
β
α
UNMODIFIED HB A_0
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220 nm
FIGURE 5a
422.000
120.000
MVOLTS
β
β1-F
β82-F
α
α1-F
β^{1-}_{82}F
β1-F-82β
β82-F-82β
α99-F-99α
FUMARYL
BIS(METHYL PHOSPHATE)
DEOXYHB
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220 nm
FIGURE 5b
10
20
30
40
50
60
70
80
MINUTES
333.000
120.000
MVOLTS
β
α
α1-F
α99-F-99α
FUMARYL
BIS(METHYL PHOSPHATE)
DEOXYHB + 2,3-DPG
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220 nm
FIGURE 5c
139.000
0.000
MVOLTS
β
β82-F
α
β82-F-82β
FUMARYL
BIS(METHYL PHOSPHATE)
COHB
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220 nm
FIGURE 5d
10
20
30
40
50
60
70
80
MINUTES

FIGURE 5

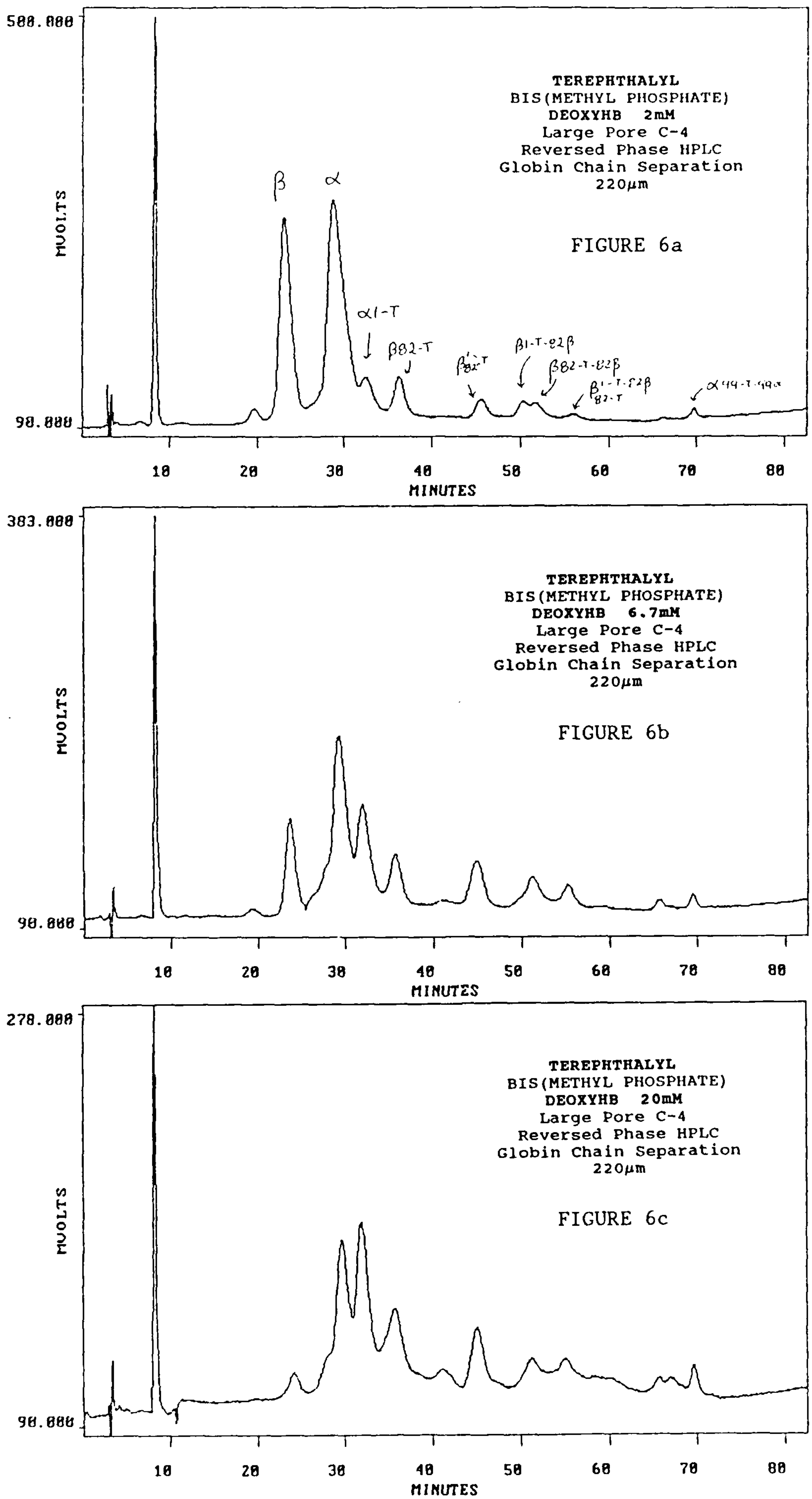

500.000
98.000
MVOLTS
β
α
α1-T
β82-T
β1-T-82β
β82-T-82β
α99-T-99α
TEREPHTHALYL
BIS(METHYL PHOSPHATE)
DEOXYHB 2mM
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220μm
FIGURE 6a
10 20 30 40 50 60 70 80
MINUTES
383.000
90.000
MVOLTS
TEREPHTHALYL
BIS(METHYL PHOSPHATE)
DEOXYHB 6.7mM
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220μm
FIGURE 6b
10 20 30 40 50 60 70 80
MINUTES
270.000
90.000
MVOLTS
TEREPHTHALYL
BIS(METHYL PHOSPHATE)
DEOXYHB 20mM
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220μm
FIGURE 6c
10 20 30 40 50 60 70 80
MINUTES

FIGURE 6

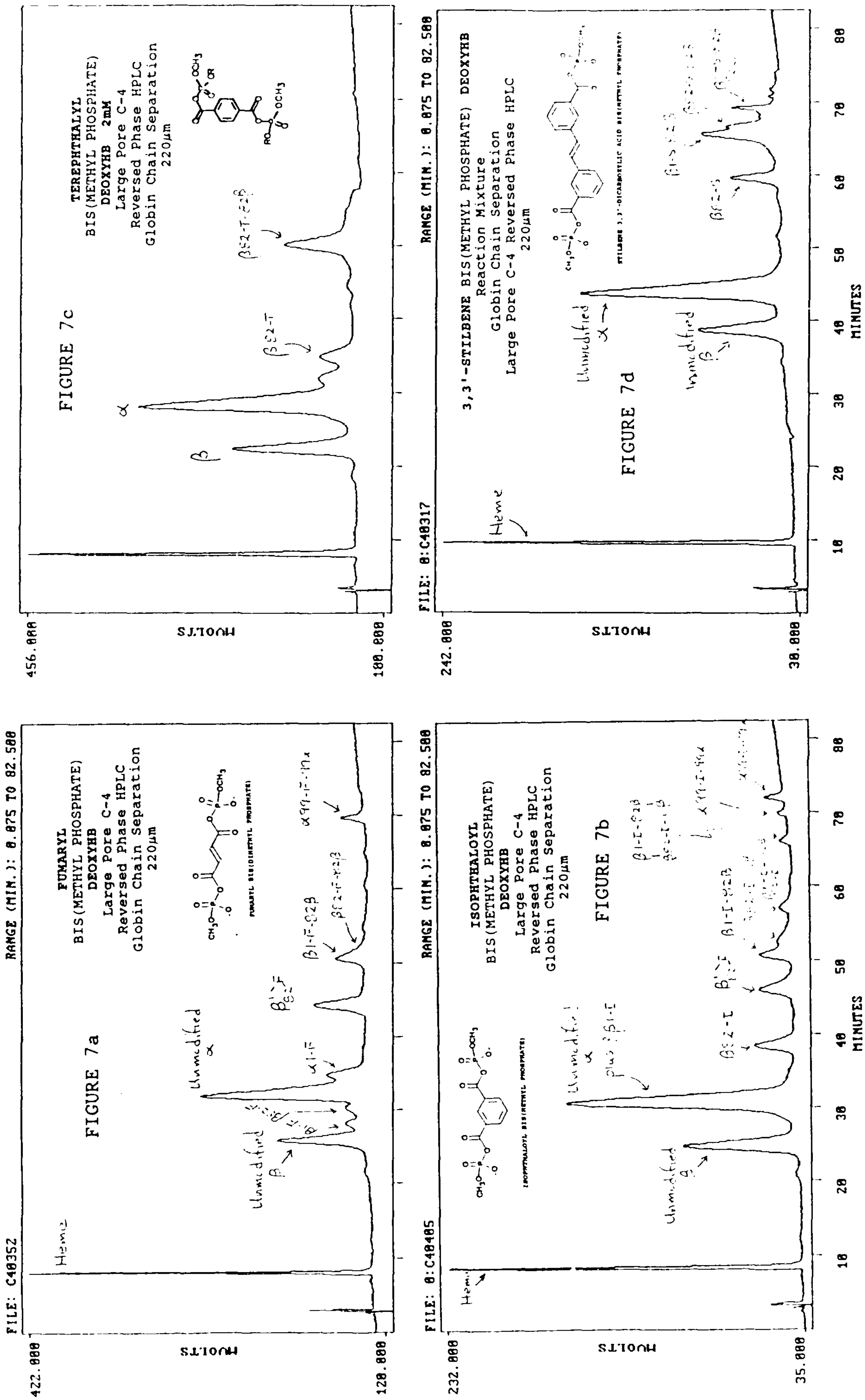


FIGURE 7c
TEREPHTHALYL
BIS(METHYL PHOSPHATE)
DEOXYHB 2mM
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220µm
FILE: 0:C40317
RANGE (MIN.): 0.075 TO 82.500
FIGURE 7d
3,3'-STILBENE BIS(METHYL PHOSPHATE) DEOXYHB
Reaction Mixture
Globin Chain Separation
Large Pore C-4 Reversed Phase HPLC
220µm
Heme
Unmodified α
Unmodified β
MINUTES
FIGURE 7a
FUMARYL
BIS(METHYL PHOSPHATE)
DEOXYHB
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220µm
FILE: C40352
RANGE (MIN.): 0.075 TO 82.500
Heme
Unmodified β
Unmodified α
FUMARYL BIS(DIMETHYL PHOSPHATE)
FIGURE 7b
ISOPHTHALOYL
BIS(METHYL PHOSPHATE)
DEOXYHB
Large Pore C-4
Reversed Phase HPLC
Globin Chain Separation
220µm
FILE: 0:C40405
RANGE (MIN.): 0.075 TO 82.500
Heme
Unmodified β
Unmodified α
MINUTES
456.000
100.000
242.000
30.000
422.000
120.000
232.000
35.000
MVOLTS

FIGURE 7

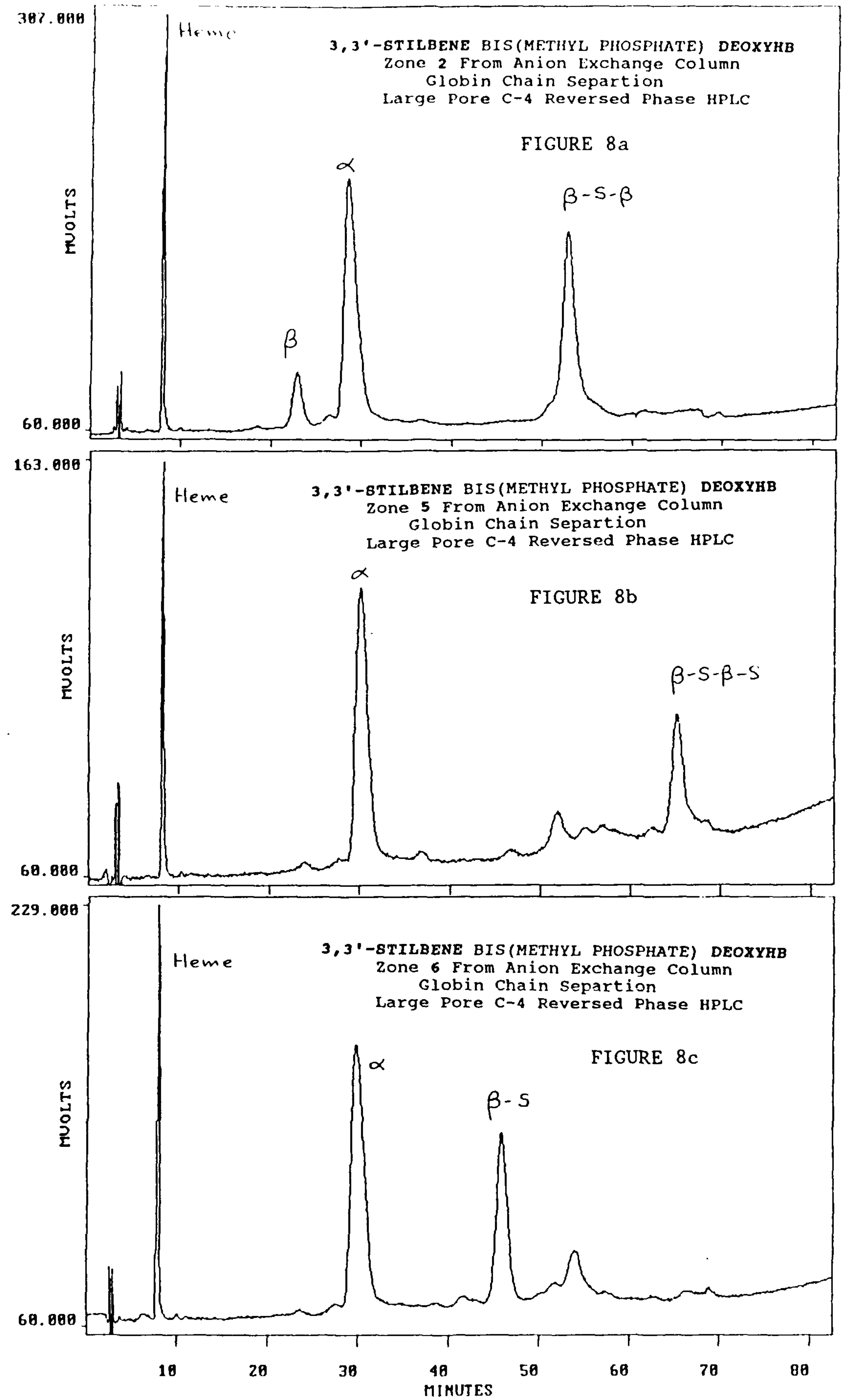

307.000
60.000
Heme
3,3'-STILBENE BIS(METHYL PHOSPHATE) DEOXYHB
Zone 2 From Anion Exchange Column
Globin Chain Separtion
Large Pore C-4 Reversed Phase HPLC
FIGURE 8a
α
β
β-S-β
MVOLTS
163.000
60.000
Heme
3,3'-STILBENE BIS(METHYL PHOSPHATE) DEOXYHB
Zone 5 From Anion Exchange Column
Globin Chain Separtion
Large Pore C-4 Reversed Phase HPLC
FIGURE 8b
α
β-S-β-S
MVOLTS
229.000
60.000
Heme
3,3'-STILBENE BIS(METHYL PHOSPHATE) DEOXYHB
Zone 6 From Anion Exchange Column
Globin Chain Separtion
Large Pore C-4 Reversed Phase HPLC
FIGURE 8c
α
β-S
MVOLTS
10
20
30
40
50
60
70
80
MINUTES

FIGURE 8

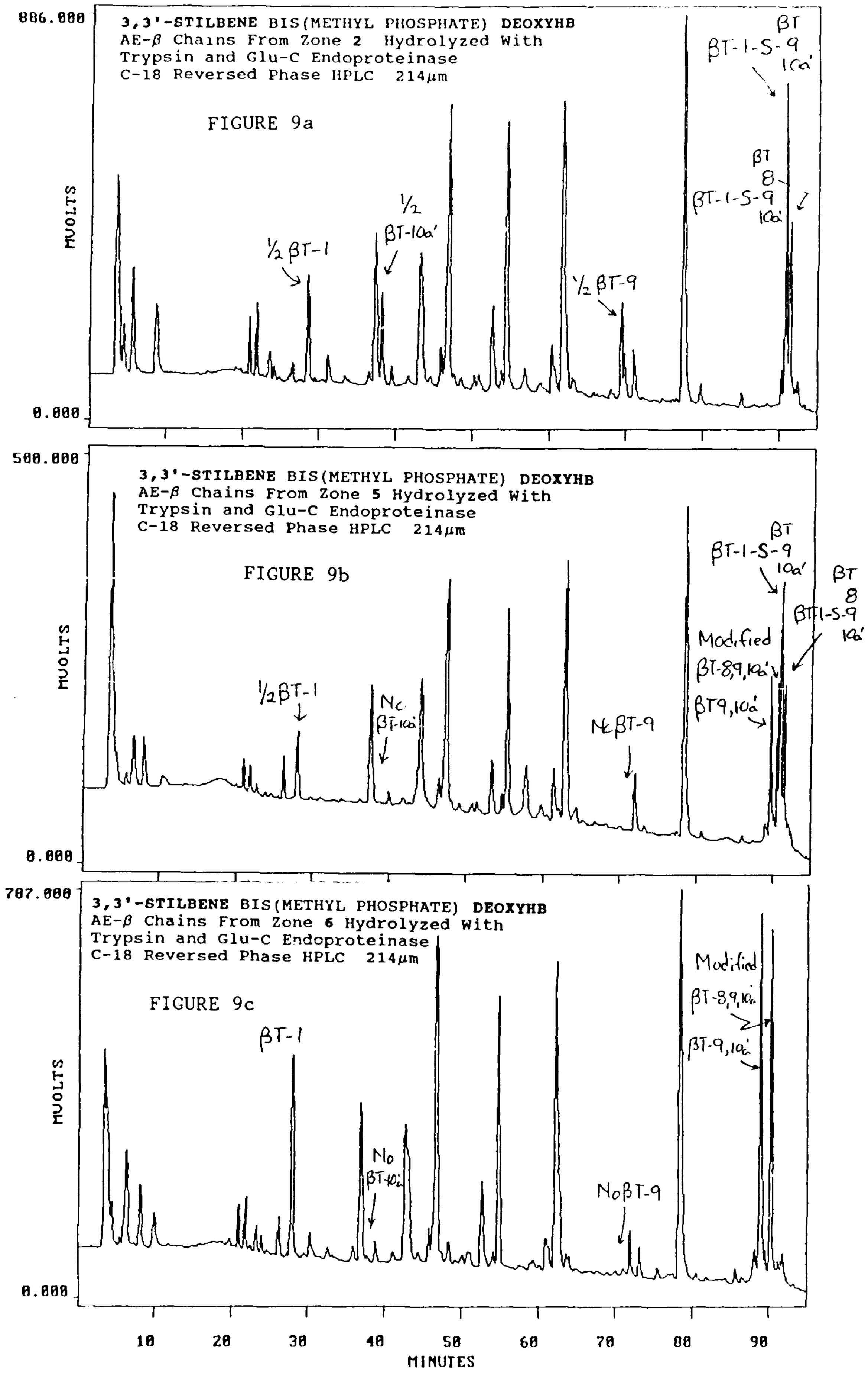
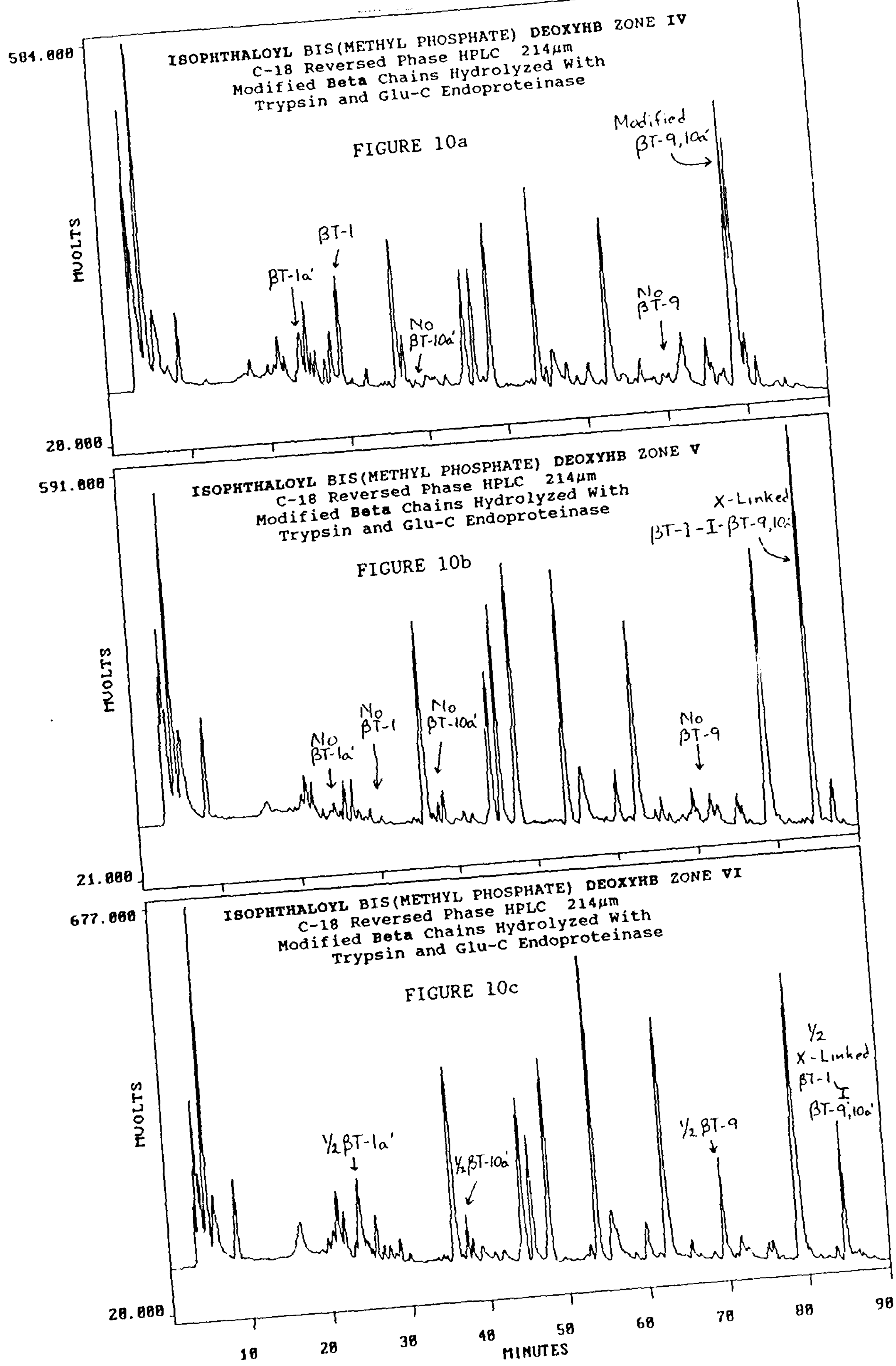

886.000
0.000
MVOLTS
3,3'-STILBENE BIS(METHYL PHOSPHATE) DEOXYHB
AE-β Chains From Zone 2 Hydrolyzed With
Trypsin and Glu-C Endoproteinase
C-18 Reversed Phase HPLC 214μm
FIGURE 9a
½ βT-1
½ βT-10a'
½ βT-9
βT βT-1-S-9 10a'
βT 8 βT-1-S-9 10a'
500.000
0.000
MVOLTS
3,3'-STILBENE BIS(METHYL PHOSPHATE) DEOXYHB
AE-β Chains From Zone 5 Hydrolyzed With
Trypsin and Glu-C Endoproteinase
C-18 Reversed Phase HPLC 214μm
FIGURE 9b
½βT-1
No βT-10a'
No βT-9
βT βT-1-S-9 10a'
βT 8 βT-1-S-9 10a'
Modified βT-8,9,10a'
βT9,10a'
707.000
0.000
MVOLTS
3,3'-STILBENE BIS(METHYL PHOSPHATE) DEOXYHB
AE-β Chains From Zone 6 Hydrolyzed With
Trypsin and Glu-C Endoproteinase
C-18 Reversed Phase HPLC 214μm
FIGURE 9c
βT-1
No βT-10a'
No βT-9
Modified βT-8,9,10a'
βT-9,10a'
10
20
30
40
50
60
70
80
90
MINUTES

FIGURE 9



ISOPHTHALOYL BIS(METHYL PHOSPHATE) DEOXYHB ZONE IV
C-18 Reversed Phase HPLC 214μm
Modified Beta Chains Hydrolyzed With
Trypsin and Glu-C Endoproteinase
FIGURE 10a
584.000
20.000
MVOLTS
βT-1a'
βT-1
No βT-10a'
No βT-9
Modified βT-9,10a'
ISOPHTHALOYL BIS(METHYL PHOSPHATE) DEOXYHB ZONE V
C-18 Reversed Phase HPLC 214μm
Modified Beta Chains Hydrolyzed With
Trypsin and Glu-C Endoproteinase
FIGURE 10b
591.000
21.000
MVOLTS
No βT-1a'
No βT-1
No βT-10a'
No βT-9
X-Linked βT-1-I-βT-9,10a'
ISOPHTHALOYL BIS(METHYL PHOSPHATE) DEOXYHB ZONE VI
C-18 Reversed Phase HPLC 214μm
Modified Beta Chains Hydrolyzed With
Trypsin and Glu-C Endoproteinase
FIGURE 10c
677.000
20.000
MVOLTS
½ βT-1a'
½ βT-10a'
½ βT-9
½ X-Linked βT-1 I βT-9,10a'
10 20 30 40 50 60 70 80 90
MINUTES

FIGURE 10

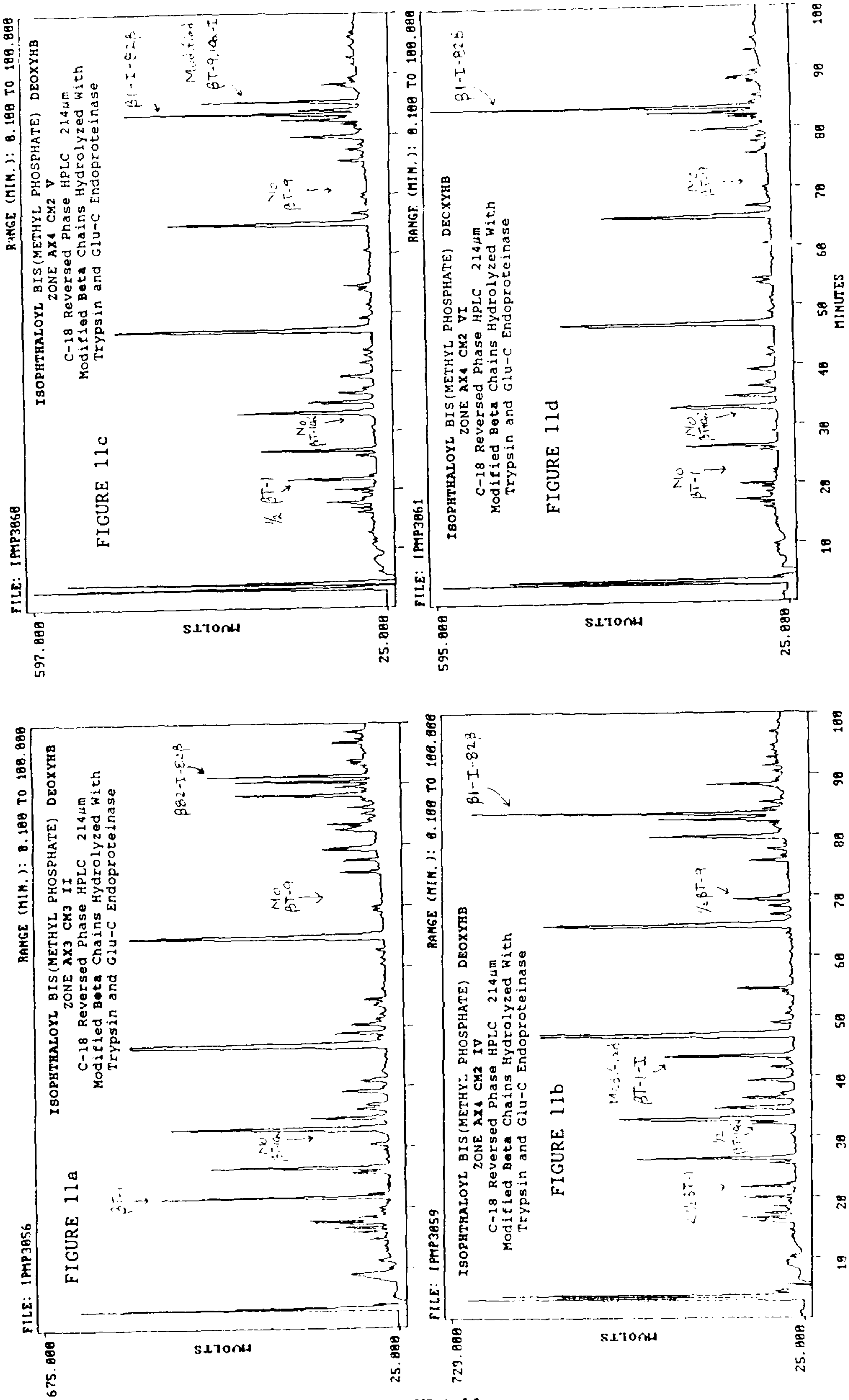
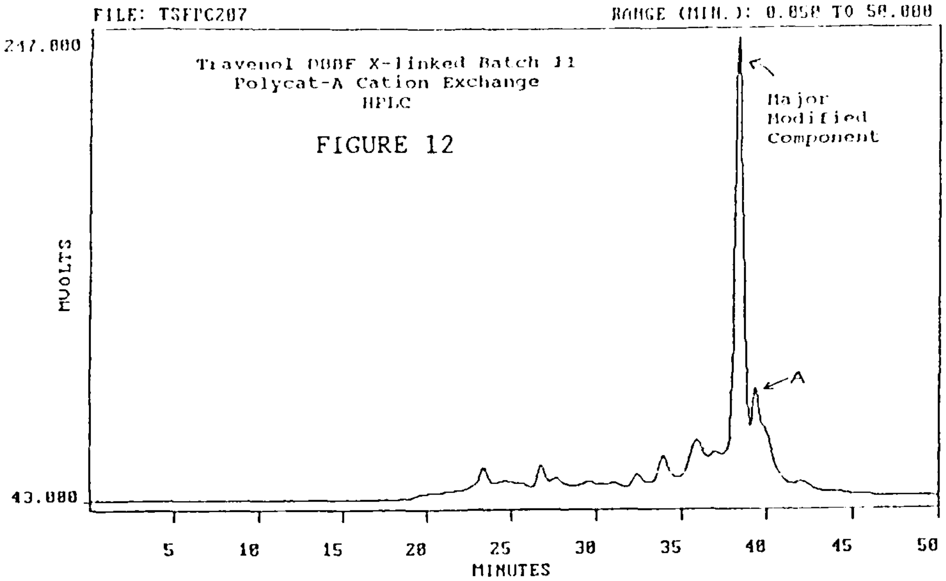


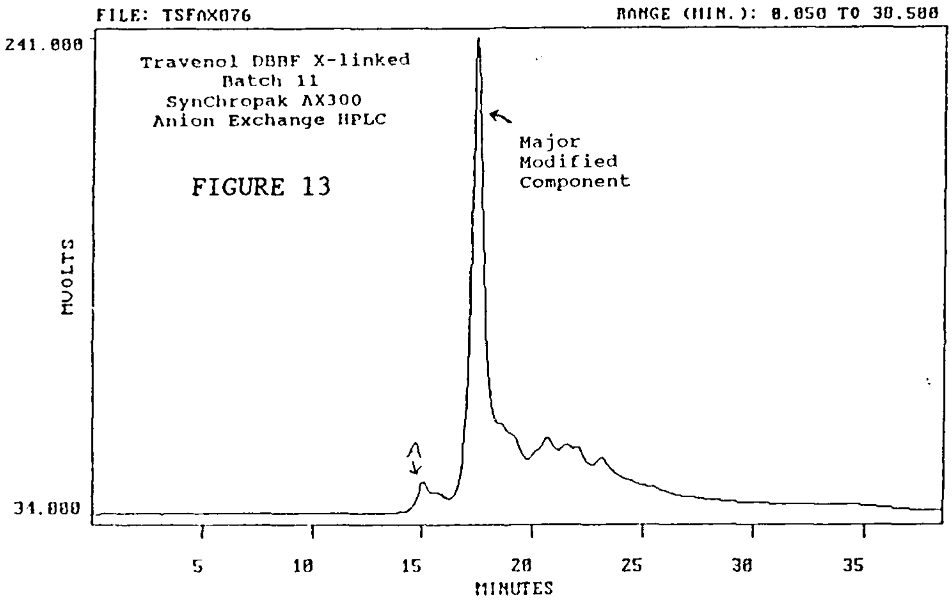
FIGURE 11a
FILE: IPMP3056
RANGE (MIN.): 0.100 TO 100.000
ISOPHTHALOYL BIS(METHYL PHOSPHATE) DEOXYHB
ZONE AX3 CM3 II
C-18 Reversed Phase HPLC 214µm
Modified Beta Chains Hydrolyzed With
Trypsin and Glu-C Endoproteinase
675.000
25.000
MVOLTS
FIGURE 11b
FILE: IPMP3059
RANGE (MIN.): 0.100 TO 100.000
ISOPHTHALOYL BIS(METHYL PHOSPHATE) DEOXYHB
ZONE AX4 CM2 IV
C-18 Reversed Phase HPLC 214µm
Modified Beta Chains Hydrolyzed With
Trypsin and Glu-C Endoproteinase
729.000
25.000
MVOLTS
MINUTES
FIGURE 11c
FILE: IPMP3060
RANGE (MIN.): 0.100 TO 100.000
ISOPHTHALOYL BIS(METHYL PHOSPHATE) DEOXYHB
ZONE AX4 CM2 V
C-18 Reversed Phase HPLC 214µm
Modified Beta Chains Hydrolyzed With
Trypsin and Glu-C Endoproteinase
597.000
25.000
MVOLTS
FIGURE 11d
FILE: IPMP3061
RANGE (MIN.): 0.100 TO 100.000
ISOPHTHALOYL BIS(METHYL PHOSPHATE) DEOXYHB
ZONE AX4 CM2 VI
C-18 Reversed Phase HPLC 214µm
Modified Beta Chains Hydrolyzed With
Trypsin and Glu-C Endoproteinase
595.000
25.000
MVOLTS
MINUTES

FIGURE 11



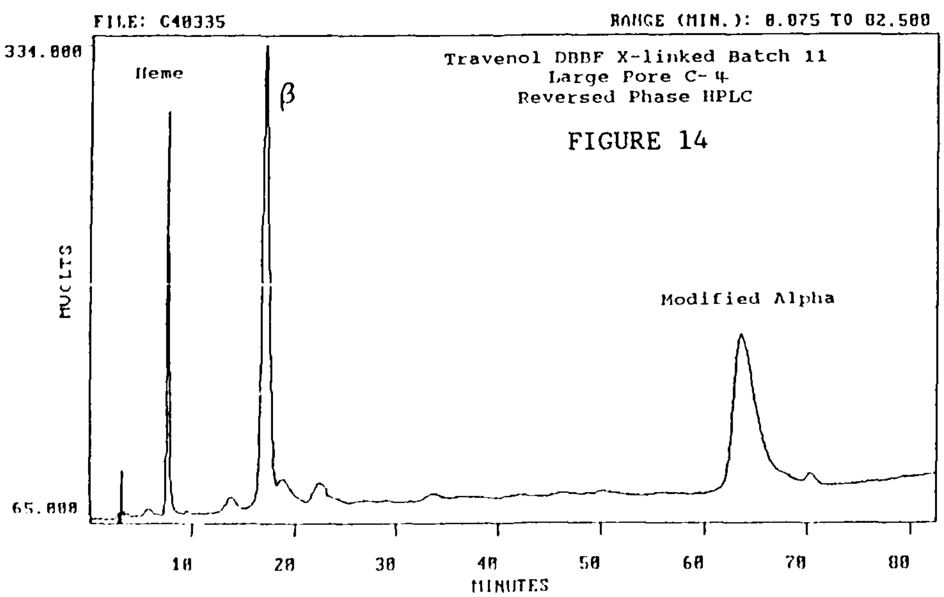
Travenol DBBF X-linked Batch 11
Polycat-A Cation Exchange
HPLC

FIGURE 12



Travenol DBBF X-linked
Batch 11
SynChropak AX300
Anion Exchange HPLC

FIGURE 13



Travenol DBBF X-linked Batch 11
Large Pore C-4
Reversed Phase HPLC

FIGURE 14

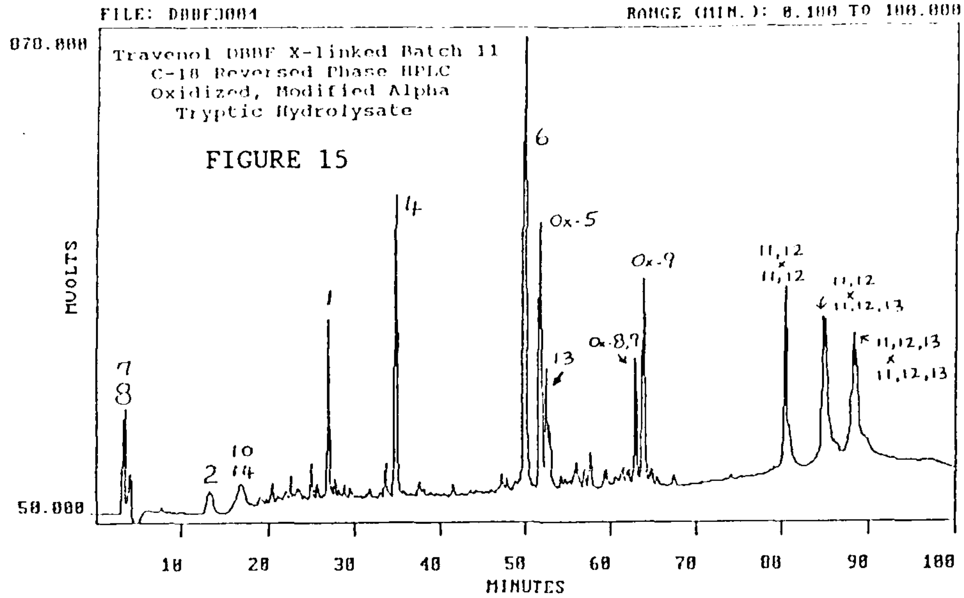


FIGURE 15

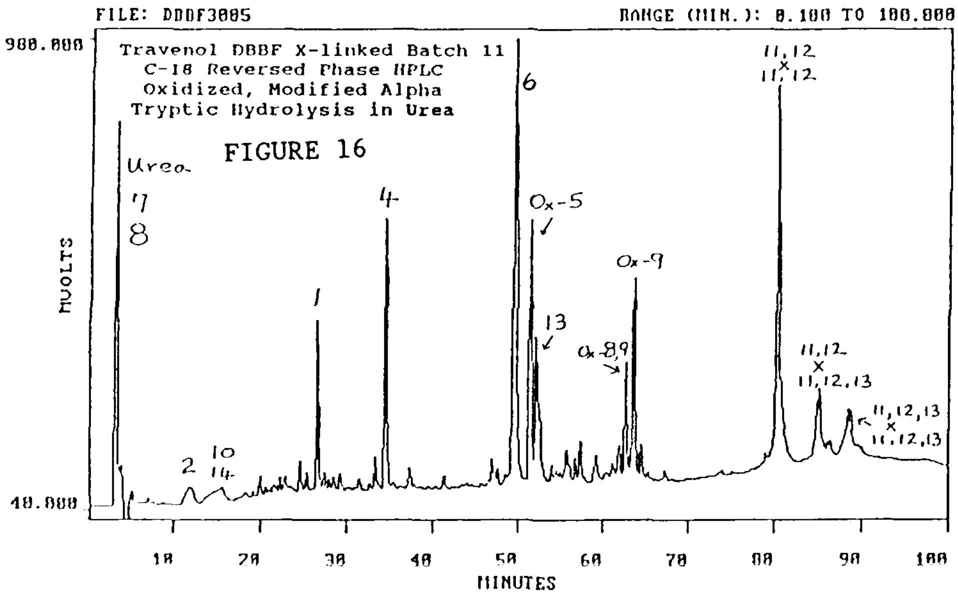


FIGURE 16

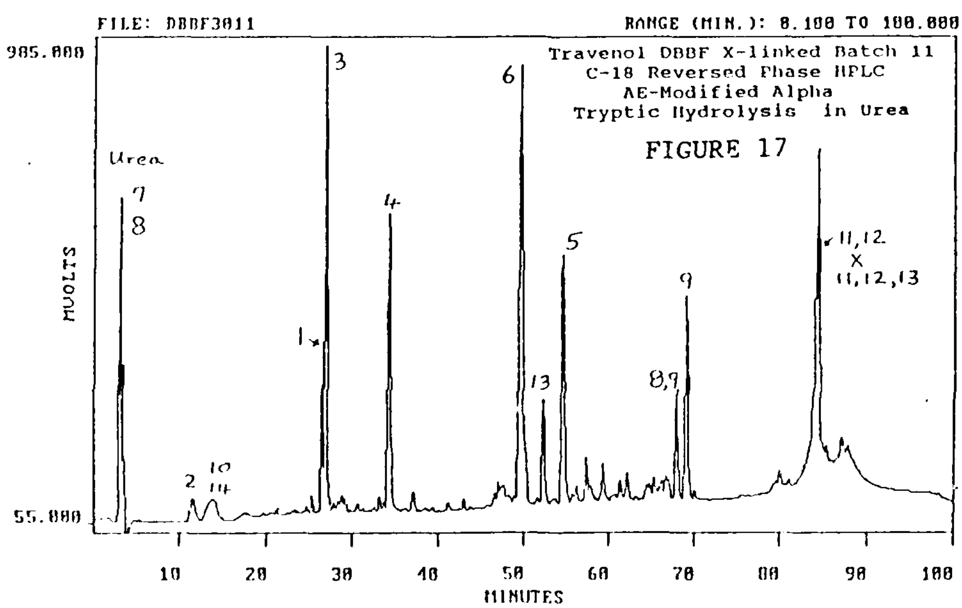
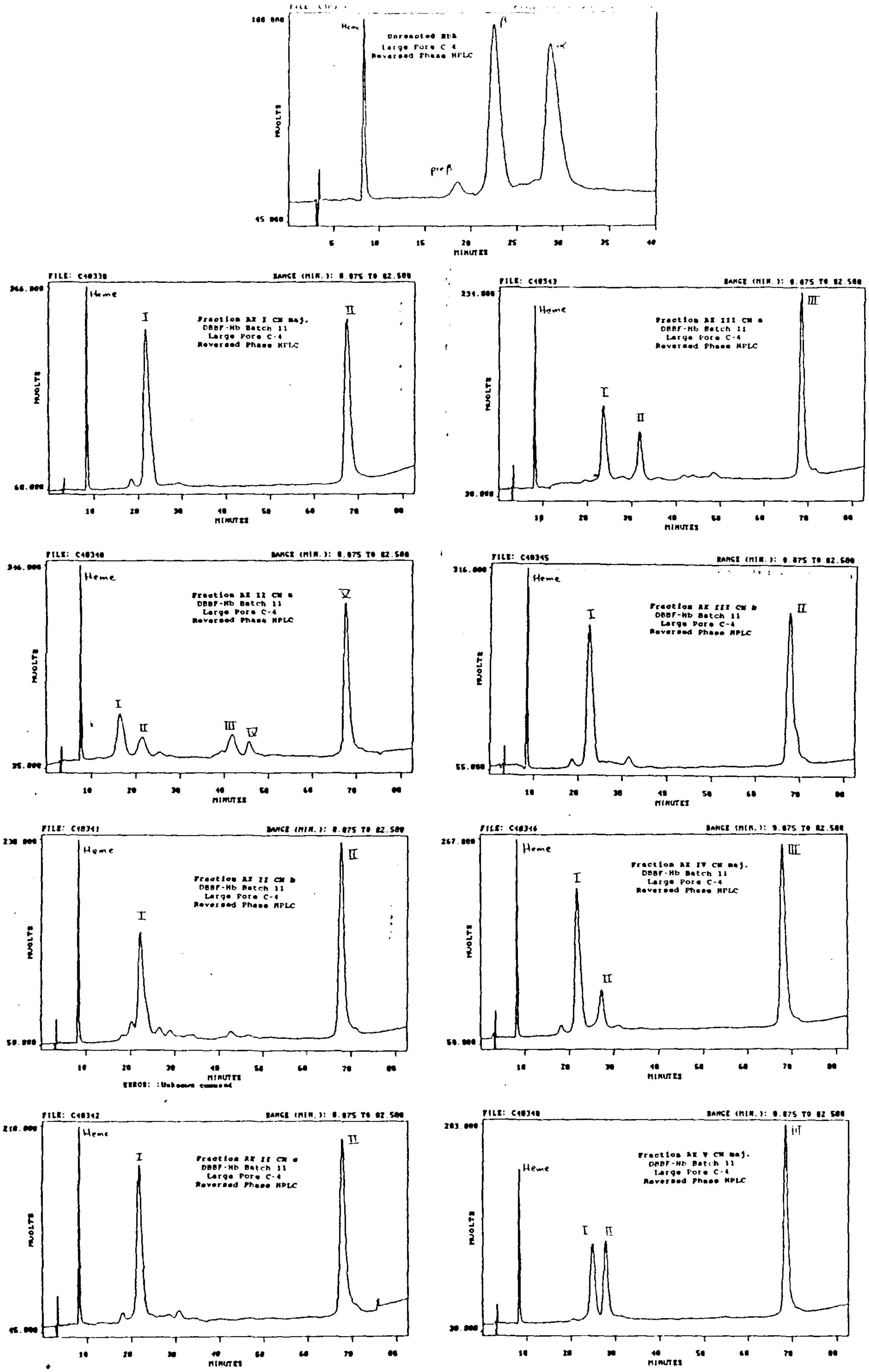


FIGURE 17



Unreacted HbA
Large Pore C 4
Reversed Phase HPLC
Heme
pre β
β
α
MVOLTS
MINUTES
Fraction AX I CN maj.
DBBF-Hb Batch 11
Large Pore C-4
Reversed Phase HPLC
Fraction AX III CN a
DBBF-Hb Batch 11
Large Pore C-4
Reversed Phase HPLC
Fraction AX II CN a
DBBF-Hb Batch 11
Large Pore C-4
Reversed Phase HPLC
Fraction AX III CN b
DBBF-Hb Batch 11
Large Pore C-4
Reversed Phase HPLC
Fraction AX II CN b
DBBF-Hb Batch 11
Large Pore C-4
Reversed Phase HPLC
ERROR: :Unknown command
Fraction AX IV CN maj.
DBBF-Hb Batch 11
Large Pore C-4
Reversed Phase HPLC
Fraction AX II CN c
DBBF-Hb Batch 11
Large Pore C-4
Reversed Phase HPLC
Fraction AX V CN maj.
DBBF-Hb Batch 11
Large Pore C-4
Reversed Phase HPLC

FIGURE 18

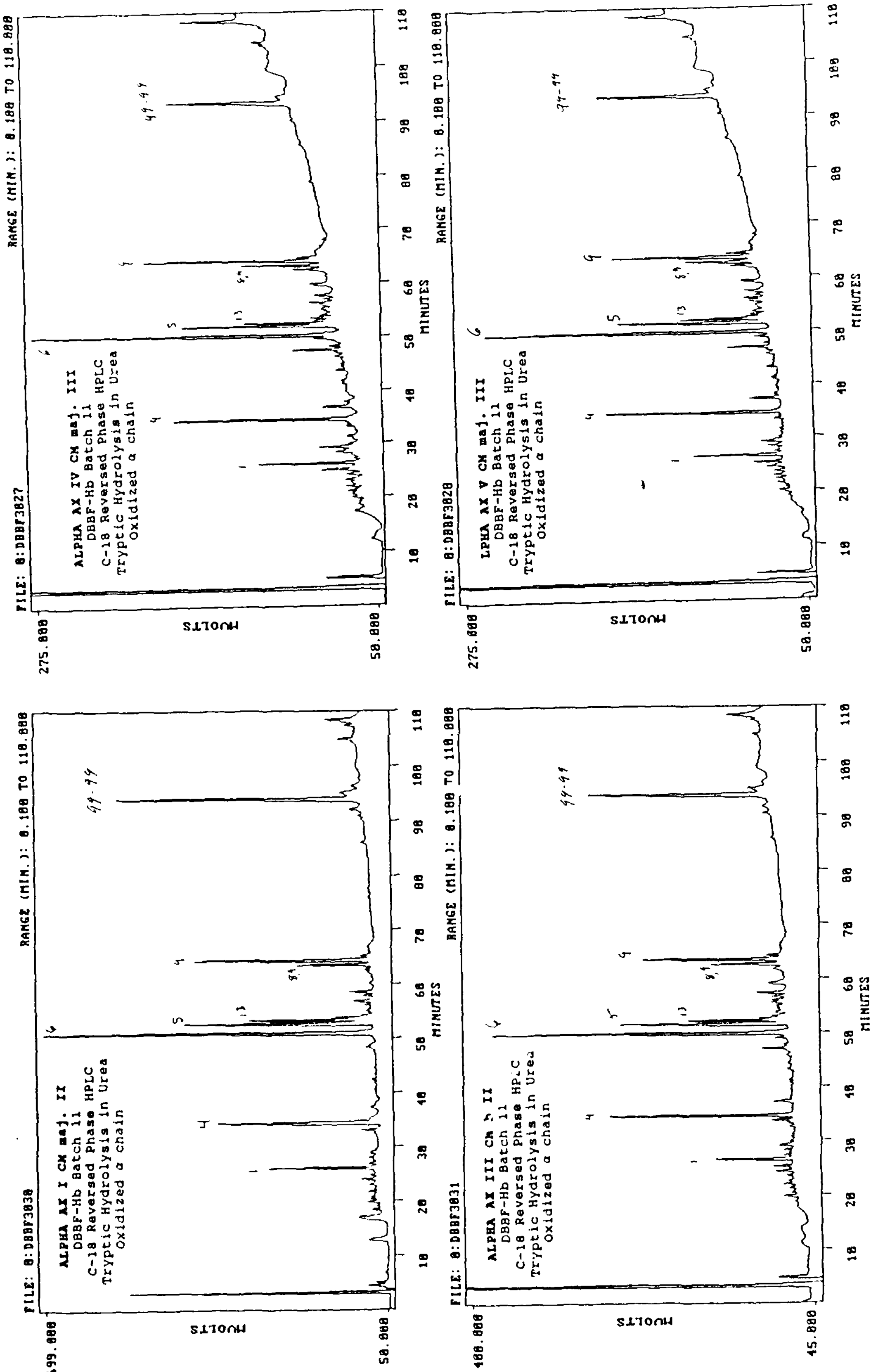

FILE: 0:DBBF3030
ALPHA AX I CM maj. II
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis in Urea
Oxidized α chain
RANGE (MIN.): 0.100 TO 110.000
MVOLTS
MINUTES
FILE: 0:DBBF3031
ALPHA AX III CM maj. II
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis in Urea
Oxidized α chain
RANGE (MIN.): 0.100 TO 110.000
MVOLTS
MINUTES
FILE: 0:DBBF3027
ALPHA AX IV CM maj. III
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis in Urea
Oxidized α chain
RANGE (MIN.): 0.100 TO 110.000
MVOLTS
MINUTES
FILE: 0:DBBF3028
LPHA AX V CM maj. III
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis in Urea
Oxidized α chain
RANGE (MIN.): 0.100 TO 110.000
MVOLTS
MINUTES

FIGURE 19

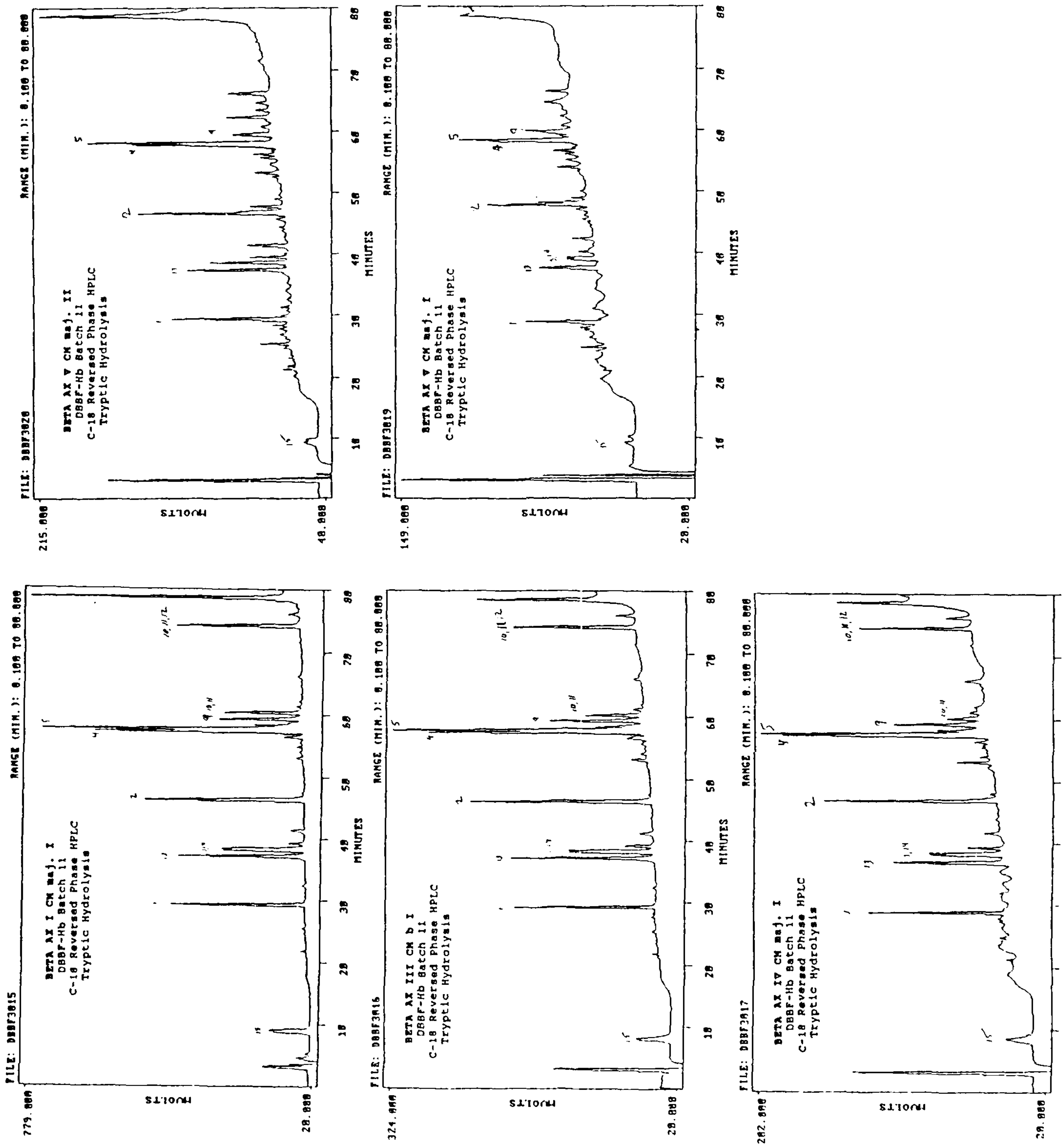

FILE: DBBF3020
RANGE (MIN.): 0.100 TO 80.000
BETA AX V CM maj. II
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis
215.000
40.000
MVOLTS
MINUTES
FILE: DBBF3019
RANGE (MIN.): 0.100 TO 80.000
BETA AX V CM maj. I
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis
149.000
20.000
MVOLTS
MINUTES
FILE: DBBF3015
RANGE (MIN.): 0.100 TO 80.000
BETA AX I CM maj. I
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis
779.000
20.000
MVOLTS
MINUTES
FILE: DBBF3016
RANGE (MIN.): 0.100 TO 80.000
BETA AX III CM b I
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis
324.000
20.000
MVOLTS
MINUTES
FILE: DBBF3017
RANGE (MIN.): 0.100 TO 80.000
BETA AX IV CM maj. I
DBBF-Hb Batch 11
C-18 Reversed Phase HPLC
Tryptic Hydrolysis
282.000
20.000
MVOLTS
MINUTES

FIGURE 20

TABLE I

Amino Acid Composition of X-linked Peptide from Tryptic + Glu-C Hydrolysis in Urea of Aminoethylated, β Chain from Zone 2 3,3'-Stilbene Bis(Methyl Phosphate) Hemoglobin

Amino Acid	Found	Expected
Asp	2.83	3
Glu	2.87	3
Ser	1.69	2
Gly	3.35	3
His	2.26	2
Thr	2.51	3
Ala	3.32	3
Pro	0.96	1
Val	2.26	2
Leu	5.94	6
Phe	2.01	2
Lys	2.02	2

Conclusion βT1-**X**-βT9,10a

TABLE II

Amino Acid Composition of Modified Peptides from Tryptic + Glu-C Hydrolysis in Urea of β Chains of Four Isophthalyl Modified Hemoglobins

Amino Acids	AX4 CM1 I	AX6 CM1 II	AX5 CM1 II	AX3 CM3 II	βT-1	βT-9,10a'	βT-1,9,10a'
Asp		2.9	3.1	3.1		3	3
Glu	2.0	1.1	3.2	1.1	2	1	3
Ser		1.7	1.9	1.8		2	2
Gly		3.2	3.3	3.2		3	3
His	0.9	0.9	1.7	1.0	1	1	2
Thr	1.0	1.9	3.1	1.9	1	2	3
Ala		3.2	3.3	2.9		3	3
Pro	1.0		1.1		1		1
Val	1.0	1.1	1.9	1.1	1	1	2
Leu	1.0	4.9	5.8	4.9	1	5	6
Phe		2.1	2.0	2.0		2	2
Lys	1.0	1.1	2.0	1.0	1	1	2
Modified Tryptic Peptide(s)	1	9 10a'	1 9 10a'	9 10a'			
Chain Structure	β1-I	β82-I	β1-I-82β	β82-I-82β			

TABLE III: STRUCTURE OF GLOBIN CHAINS FROM FUMARYL BIS (METHYL PHOSPHATE) MODIFIED HEMOGLOBINS

Anion Zone #	Cation Zone #	Chain Zone #	Chain and Modification
A. Hemoglobins From FMP Treated Deoxyhemoglobin			
AX-1	CM-1	I	β-unmodified
"	"	II	α-unmodified
AX-2	CM-1	I	α-unmodified
"	"	II	$\beta_1 1$-$\beta_2 82$
AX-3b	CM-1	I	β-unmodified
"	"	II	α-unmodified
"	"	III	$\beta_1 1$-$\beta_1 82$
"	"	IV	$\beta_1 1$-$\beta_2 82$
"	"	V	α-modification?
"	"	VI	$\alpha_1 99$-$\alpha_2 99$
AX-3b	CM-2	I	α-unmodified
"	"	II	$\beta_1 82$-$\beta_2 82$
AX-3b	CM-3	I	β-unmodified
"	"	II	$\alpha_1 1$-Fumarate?
"	"	III	$\beta_1 82$-$\beta_2 82$
AX-3c	CM-1	I	$\beta_1 1$-Fumarate
"	"	II	α-unmodified
AX-3c	CM-2	I	β-unmodified
"	"	II	β-modification?
"	"	III	α-unmodified
"	"	IV	α-modification?
"	"	V	$\alpha_1 99$-$\alpha_2 99$
AX-4	CM-1	I	α-unmodified
"	"	II	$\beta_1 1$-$\beta_1 82$
AX-5	CM-1	I	$\beta_1 82$-Fumarate
"	"	II	α-unmodified
B. Hemoglobins From FMP Treated Deoxyhemoglobin with 2,3-DPG			
AX-1	CM-1	I	β-unmodified
"	"	II	α-unmodified
AX-2	CM-1	I	β-unmodified
"	"	II	$\alpha_1 99$-$\alpha_2 99$
AX-3	CM-1	I	β-modified?
"	"	II	α-modified?
C. Hemoglobins From FMP Treated Carbonmonoxyhemoglobin			
AX-1	CM-1	I	β-unmodified
"	"	II	α-unmodified
AX-2	CM-1	I	α-unmodified
"	"	II	$\beta_1 82$-$\beta_2 82$

TABLE IV: STRUCTURE OF GLOBIN CHAINS FROM ISOPHTHALYL BIS(METHYL PHOSPHATE) MODIFIED HEMOGLOBINS

Anion Zone #	Cation Zone #	Chain Zone #	Chain and Modification
A. Hemoglobins from IPMP Treated Deoxyhemoglobins			
AX-1	CM-1	I	β-unmodified
"	"	II	α-unmodified
AX-2	CM-1	I	α-unmodified
"	"	II	β1-I-82β
AX-3	CM-1	I	β-unmodified?
"	"	II	α-unmodified
"	"	III	β1-I-82β
"	"	IV	β1-I-82β1-I
"	"	V	$\beta^{1\text{-}I\text{-}82}_{82\text{-}I\text{-}1}\beta$
"	"	VI	α99-I-99α
"	"	VII	α99-I-127α
AX-3	CM-2	I	α-unmodified
"	"	II	β82-I-1β82-I
AX-3	CM-3	I	α-unmodified
"	"	II	β82-I-82β
AX-4	CM-1	I	β1-I
"	"	II	α-unmodified
AX-4	CM-2	I	β-unmodified
"	"	II	α-unmodified
"	"	III	β1-I-82β
"	"	IV	β1-I-82-β1-I
"	"	V	β82-I-1β82-I
"	"	VI	$\beta^{1\text{-}I\text{-}82}_{82\text{-}I\text{-}1}\beta$
"	"	VII	α99-I-127α
AX-4	CM-3	I	α-unmodified
"	"	II	β1-I-82β
AX-4	CM-4	I	β82-I
"	"	II	α-unmodified
"	"	III	β82-I-82β
AX-4	CM-5	I	β82-I
"	"	II	α-unmodified
AX-5	CM-1	I	α-unmodified
"	"	II	$\beta^{1}_{82}{>}\mathrm{I}$
"	"	III	β82-I-1β82-I
AX-6	CM-1	I	α-unmodified
"	"	II	β82-I
B. Hemoglobins from IPMP Treated Carbonmonoxyhemoglobin			
AX-2	CM-1	I	α-unmodified
"	"	II	β82-I-82β

TABLE V

SUMMARY OF STUDIES ON HEMOGLOBIN COMPONENTS FROM REACTION OF DEOXYHEMOGLOBIN WITH 3,3'STILBENE BIS(METHYL PHOSPHATE)

Hb	SDS-PAGE	Globin Chains	Peptide Pattern	Modified Chain
1	no x-link	unmodified	no modification	none
2	x-linked	β-modified	$\frac{1}{2}\beta$T-1, T-9, T10a'	$\beta_1$1-S-82β_2
4	x-linked	β-modified	no βT-9 or T-10a'	$\beta_1$82-S-82β_2
5	x-linked	β-modified	$\frac{1}{2}\beta$T-1 no βT-9 or T-10a'	$\beta_2$82-S-1$\beta_1$82-S
6	no x-link	β-modified	no βT-9 or T-10a'	$\beta_1$82-S & $\beta_2$82-S

TABLE VI

SUMMARY OF MODIFIED GLOBIN CHAINS
OBTAINED WITH DICARBOXYLIC BIS(METHYL PHOSPHATE)
REAGENTS

Fumaryl	Isophthalyl	3,3'-Stilbene
β1-F	β1-I	
β82-F	β82-I	β82-S
α1-F		
$\beta^{1}_{82}{>}\mathrm{F}$	$\beta^{1}_{82}{>}\mathrm{I}$	
β1-F-82β	β1-I-82β	β1-S-82β
β82-F-82β	β82-I-82β	β82-S-82β
	$\beta^{1\text{-}I\text{-}82}_{82\text{-}I}\beta$	$\beta^{1\text{-}S\text{-}82}_{82\text{-}S}\beta$
	$\beta^{1\text{-}I\text{-}82}_{82\text{-}I\text{-}1}\beta$	
α99-F-99α	α99-I-99α	
	α99-I-139α	

TABLE VIIA

DISTANCES BETWEEN
β1Val-NH_2 AND β82Lys-NH_2
IN OXY- & DEOXY-Hb

Residues	Distances Å Deoxy-	Oxy-
$\beta_1$1Val--$\beta_2$1Val	18.4	19.9
$\beta_1$1Val--$\beta_2$82Lys	11.5	15.5
$\beta_1$82Lys--$\beta_2$82Lys	9.3	10.7
$\beta_1$1Val--$\beta_1$82Lys	9.9	5.4

TABLE VIIB

DISTANCES BETWEEN NITROGENS
FOR SELECTED CROSS-LINKERS

Cross-linker	Distances Å
Fumaryl	6.1
Isophthalyl	7.3
Terephthalyl	7.5
3,3'-Stilbene	13.2
DIDS	16

TABLE VIII

FUNCTIONAL PROPERTIES OF
MAJOR MODIFIED HEMOGLOBINS

P_{50} (n_{50})

	Fumaryl	Isophthalyl	3,3'-Stilbene
$\alpha_2\beta 1\text{-X-}82\beta$		17.0 (2.3) 17.8 (2.7)	4.2 (2.5)
$\alpha_2\beta^{1\text{-X-}82}_{82\text{-X-}1}\beta$		22.5	
$\alpha_2\beta^{1}_{82}{>}X$		9.4 (2.6)	
$\alpha_2(\beta 82\text{-X})_2$	9.8 (2.3)	8.7 (2.8)	
$\alpha_2(\beta 1\text{-X})_2$		6.9	

Conditions: 50 mM bis Tris, 0.1 M Cl^-, pH 7.4, 25° C, 55 μM Heme
Normal Unmodified Hb A: P_{50}= 4.9, n_{50}= 2.9

TABLE IX
SUMMARY OF CHROMATOGRAPHIC STUDIES OF
BATCH 11 DBBF X-LINKED HEMOGLOBIN

Fraction Designations and Relative %							Conclusions
AX300		CM300		Chain		Peptide	
Frac. #	%	Frac. #	%	Frac	%	Pattern	
PreAXI	5%						Unmodified Hb A
AXI	45%	CMmaj.	100%	I	50%	normal B	Unmodified B
				II	50%	a99-F-99a	a99 cross-linked
AXII	10%	CMa	33%	I	22%		PreB
				II	10%		Unmodified B
				III	10%		Modified B
				IV	7%		Modified B
				V	50%		a99 cross-linked
		CMb	23%	I	25%		Unmodified B
				minors	25%		Mixture of Modified B
				II	50%		a99 cross-linked
		CMc	44%	I	40%		Unmodified B
				minors	10%		Mixture of Modified B
				II	50%		a99 cross-linked
AXIII	15%	CMa	52%	I	25%		? B1-F
				II	15%		Unmodified B
				minors	10%		Mixture of Modified B
				III	50%		a99 cross-linked
		CMb	48%	I	40%	normal B	Unmodified B
				minors	10%		Mixture of Modified B
				II	50%	a99-F-99a	a99 cross-linked
AXIV	10%	CMmaj.	65%	I	40%	normal B	Unmodified B
				II	10%		? B82-F
				III	50%	a99-F-99a	a99 cross-linked
AXV	5%	CMmaj.	75%	I	25%	altered B	? B1-F
				II	25%	altered B	? B82-F
				III	50%	a99-F-99a	a99 cross-linked
AXVI	10%	CMmaj.	75%	Not studied further			